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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Purpose: Alternative splicing is responsible for producing several products from a single transcript and can cause pathogenic changes in RNA in neurodegenerative disease. This proposal tests the hypothesis that regulation of normal splicing is disrupted in Parkinson's disease (PD). Scope: Experiments are designed to determine splicing products in the brain and blood of experimental MPTP models of PD and the blood of newly diagnosed PD patients, who are not yet on dopamine therapy. The overall goal is to use splice variants as biomarkers to identify individuals at risk for PD. To date, we have identified and quantified alternatively spliced transcripts for several candidate genes in MPTP models of PD. We have also obtained IRB permission to study splicing factors in the blood of newly diagnosed PD patients. Major Findings: Mice treated acutely and chronically with MPTP show a shift in the ratio of FosB, RGS9, AChe and Ania6 splice variants in the striatum and blood. Gene expression (in situ hybridization) studies are in progress to localize the variants in the brain. Progress in the second year includes 3 abstracts, one published article, a second article submitted and an article in preparation.

15. SUBJECT TERMS
Pre-mRNA splicing, MPTP, Parkinson's disease, gene expression, alternative splicing, intron, exon

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Introduction

In humans, non-human primates and mice, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes pathological damage similar to that observed in Parkinson's disease (PD) (Langston et al., 1984). The active metabolite of MPTP is MPP+ and this blocks the respiratory chain in mitochondria resulting in increased free radicals and ultimately cell death. Molecular changes in gene expression occur rapidly in response to environmental stimuli, and these changes can occur in the peripheral blood circulation as well as in the brain (Bas et al., 1993). A few alternative splice products of different genes have been identified in experimental parkinsonism and confirmed in Parkinson's disease (PD) patients (Tekumalla et al., 2001). Such variants are produced in different proportions in healthy individuals, which means that alternative splice variants could be useful biomarkers of the disease state. During this past year we have identified several changes in the ratios of splice isoforms of mRNA transcripts in the brain and blood of mice treated chronically or acutely with MPTP. We have published or submitted for publication some of these findings and in addition, are carrying out gene expression studies to localize the aberrant spice products.

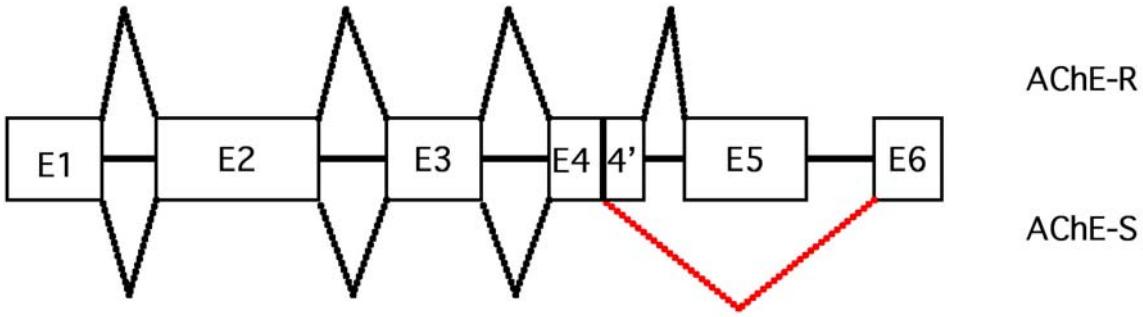
Body

We have made good progress during the second year of this grant and we outline our accomplishments under each Objective of the original Statement of Work. We have identified spice variants of rgs9 and fosB that are rapidly changed in the blood and brain of mice treated acutely or chronically with MPTP. We have also identified splice variants of ache, ania6 and ndufs4 that change in the brain or blood of treated mice. We outline our key research accomplishments below.

Objective 1: To identify abnormal splice variants of genes involved in the development and progression of Parkinsonism in the brain and blood of chronic rodent models of PD.

We have completed all parts of Objective 1 in the Statement of Work. Published results and a submitted manuscript are attached to this report. The new data obtained over the past year includes splice variants that were quantified and normalized to 18S rRNA. By quantifying in this manner we are able to examine the steady state levels of RNA, taking into account changes that occur due to transcription and RNA stability, in addition to those that occur because of a dysregulation of splicing. We now express the data as splice variant1/ splice variant2, splice variant1/18S rRNA and splice variant2/18S rRNA. This has allowed us to observe additional changes in the amounts of the fosB and rgs9 splice variants after acute and chronic MPTP treatment. In particular, we believe that we have identified changes in the pre-mRNA splicing of the fosB and rgs9 transcripts in the blood of mice, such that the ratio of fosB/18S rRNA and rgs9-2/18S rRNA transcripts decreased after acute MPTP treatment, and the fosB/18S rRNA and rgs9-2/rgs9-1 transcript ratios decreased after chronic MPTP/probenecid treatment. These data suggest that changes in the ratio of splice variants of fosB and rgs9 in the blood may prove to be useful as biomarkers to detect disease dcevelopment in humans, since they indicate the immediate and long-term responses to the physiological stress. In addition, we have quantified the FosB and RGS9 proteins in the brains of mice treated chronically with MPTP/probenecid. The results from those studies showed there was an increase in FosB/tubulin, FosB/tubulin, RGS9-2/RGS9-1, RGS9-1/tubulin and RGS9-2/tubulin in the striatum in the MPTP-treated mice euthanized 3 days post-treatment and in FosB/FosB 3 weeks post-treatment. The results from the fosB and rgs9 splice variants in the brain are presented in the Brain Research paper that is in press and attached in the appended materials. The results from the fosB and rgs9 splice variants in the blood are presented in the Neuroscience paper that is submitted for review and attached in the appended materials.

In addition to the above splice variants we have new data on three more splice variants that is presented below.


E1
E2
E3
E4
4'
E5
E6
AChE-R
AChE-S

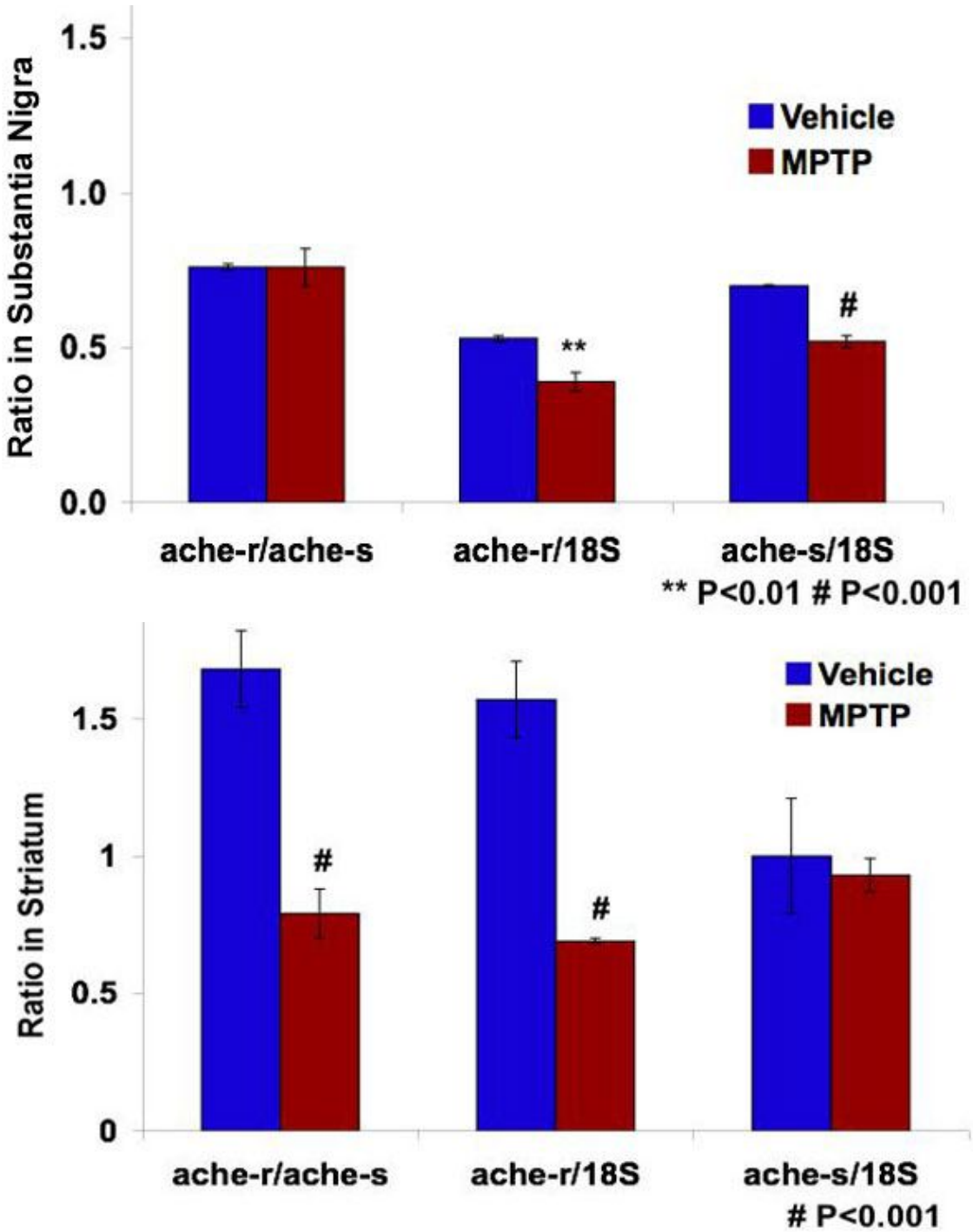


Fig. 1. Ache splice variants in the brains of mice after acute MPTP treatment. The mice were euthanized 3 days after treatment.

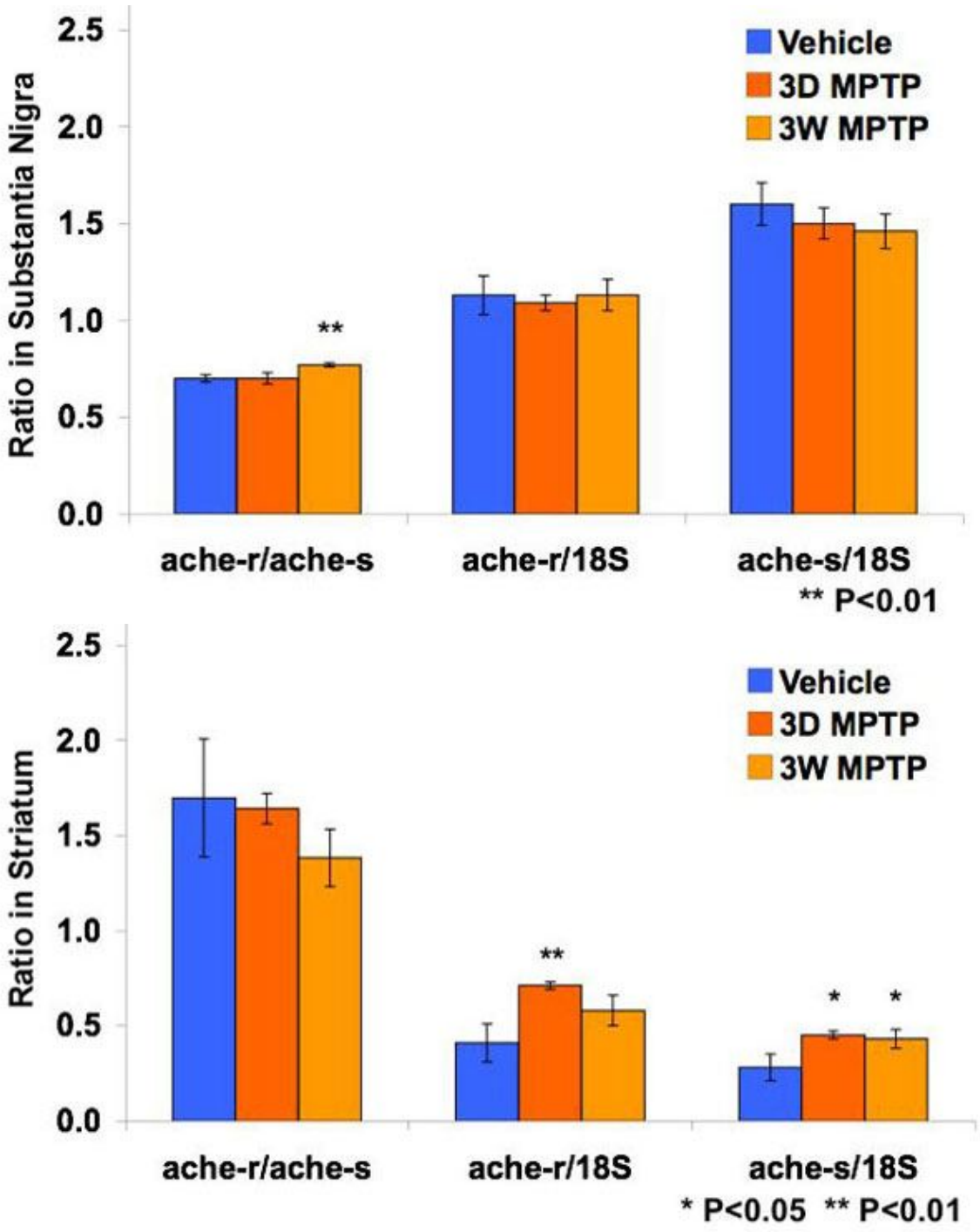


Fig. 2. Ache splice variants in the brains of mice after chronic MPTP treatment. The mice were euthanized 3 days (D) or 3 weeks (WK) after toxin administration.

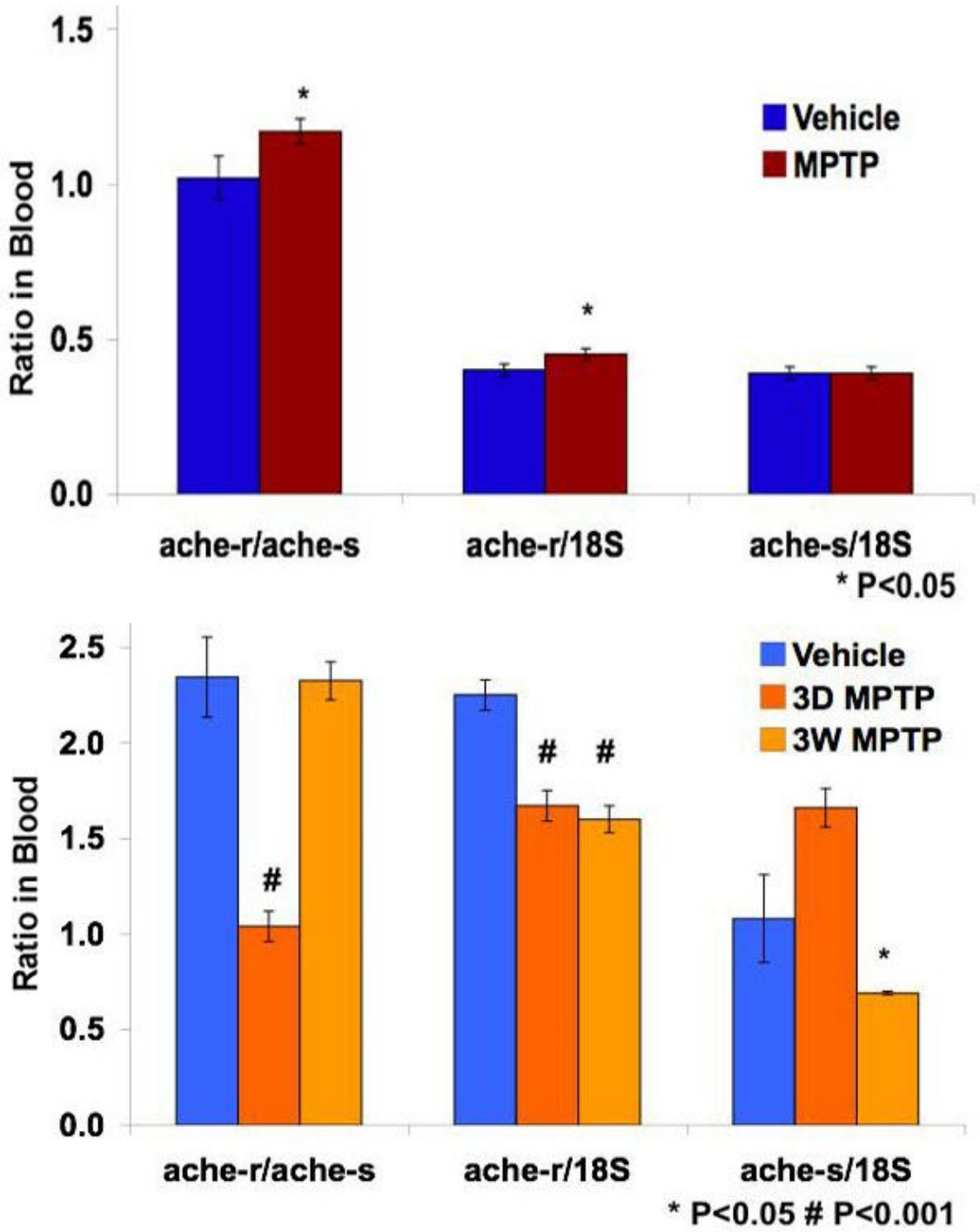


Fig. 3. Ache splice variants in the blood of chronic MPTP-treated mice. The mice were euthanized 3 days after acute MPTP treatment (top panel), or 3 days (D) or 3 weeks (WK) after chronic MPTP administration (bottom panel).

The results presented in figures 1-3 show that there are significant changes in the ache splice variants in the blood and brain after MPTP treatment. Specifically, the ratio of ache-r/ache-s was increased in the subsantia nigra 3 weeks after chronic MPTP treatment compared to vehicle (Fig. 2), but not changed after acute MPTP administration (Fig. 1). The ratio of ache-r/ache-s was decreased in the striatum after acute MPTP treatment compared to vehicle (Fig. 1), but not changed after chronic MPTP administration (Fig.2). In the blood, there was a significant increase in the ratio of ache-r/ache-s after acute MPTP treatment, but a decrease 3 days after chronic treatment (Fig. 3). Figure 4 illustrates the gene expression for ache-r from the midbrains of a chronic MPTP- and saline- treated mice. There is no difference in the expression of the ace-r factor between the two treatments. These findings suggest that changes in ache splicing accompany the development of Parkinsonism and that screening for splice variants in the blood may be a useful indicator of changes that may predispose individuals to the development of PD.

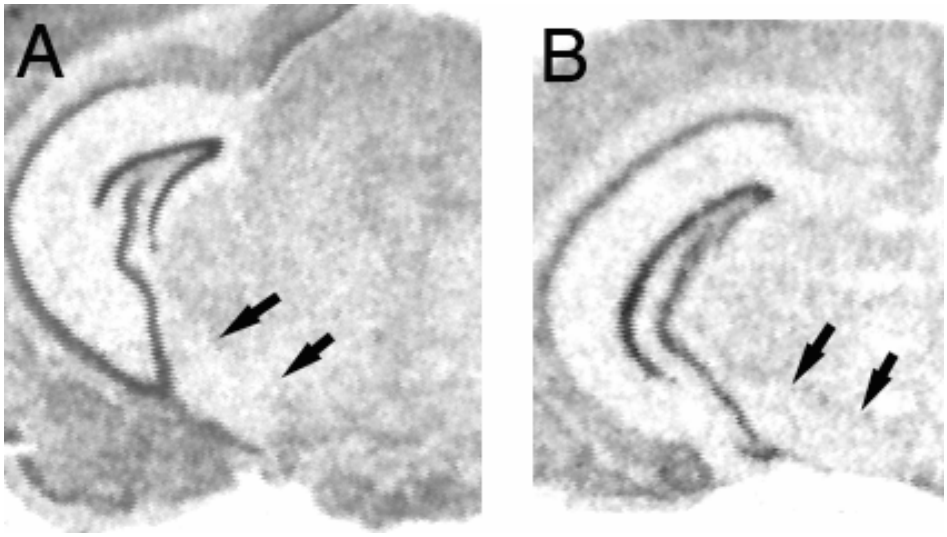
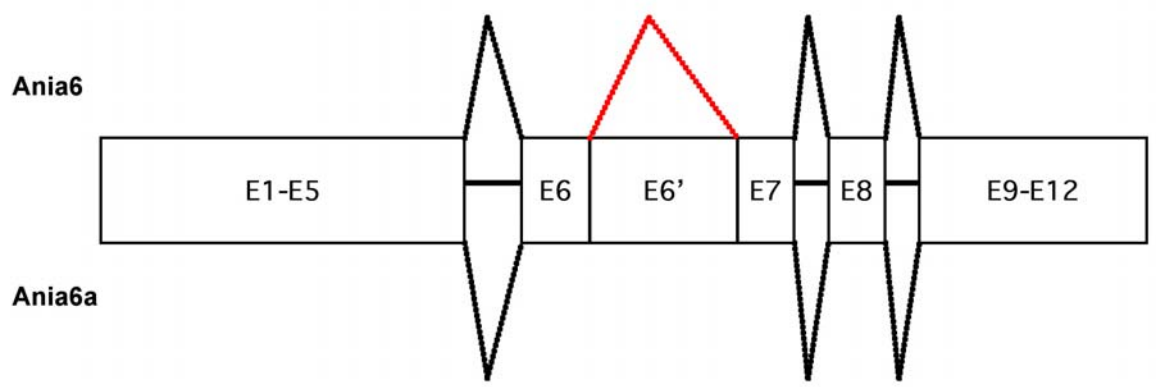
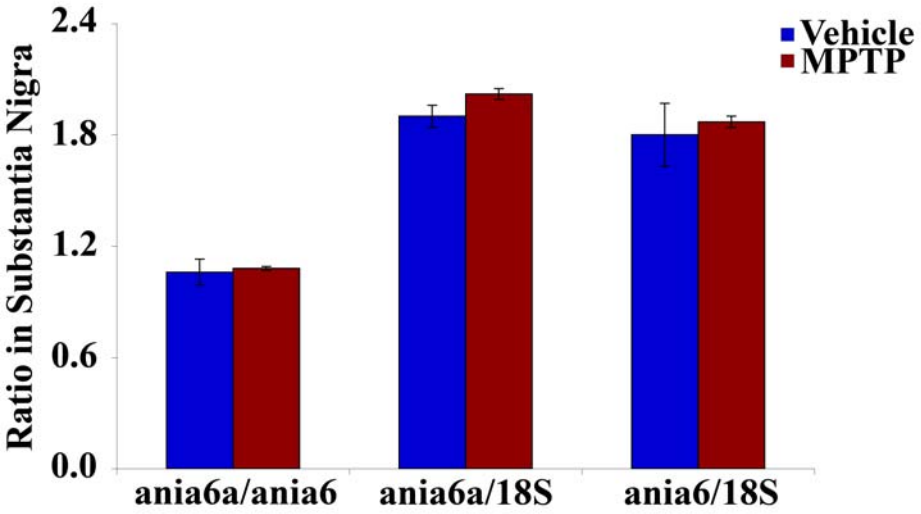


Fig. 4. Examples of film autoradiograms depicting the gene expression for the ache-r splice variant in the midbrain of a mouse treated chronically with (A) MPTP or (B) saline. Arrows point to the expression of this factor in the SNpc.

We have also investigated the splice variants of ania 6 which has two splice variants ania6 and ania6a due to the inclusion or exclusion of exon 6' (Fig. 5, top panel). The Ania

6 gene encodes a cyclin, and its expression is induced in the striatum by dopamine stimulation (Berke et al., 2001). The Ania 6 protein contains a carboxyl-terminus with an arginine/serine-rich domain that is absent from the Ania 6a protein. This region of the protein is important for the association between the Ania 6 protein and splicing factors in the nucleus.





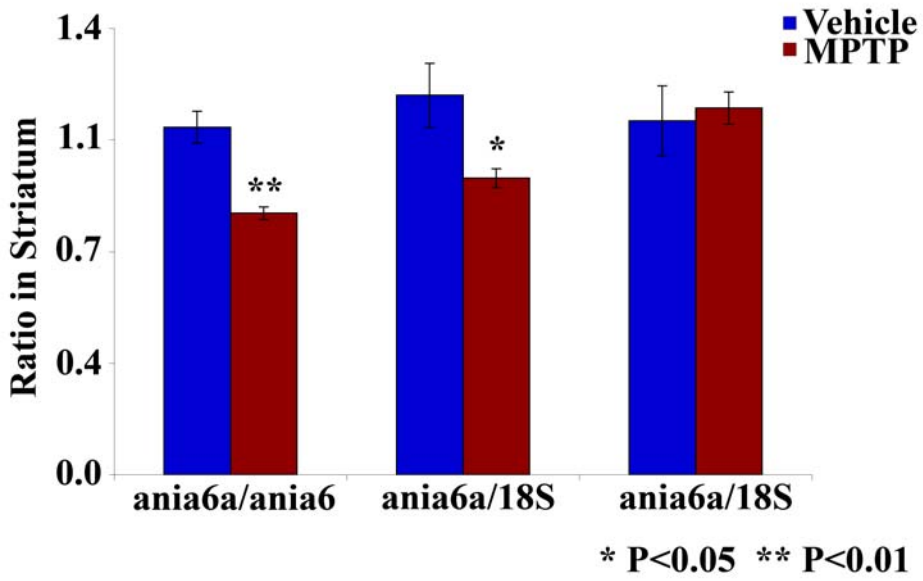


Fig. 5. Ania 6 splice variants in the brains of mice after acute MPTP treatment. The mice were euthanized 3 days after treatment.

We observed a significant decrease in the ratio of ania6a/ania6 and ania6a/ 18S in the striatum of acutely-treated mice that were euthanized 3 days post-MPTP/probenecid treatment (Fig. 5).

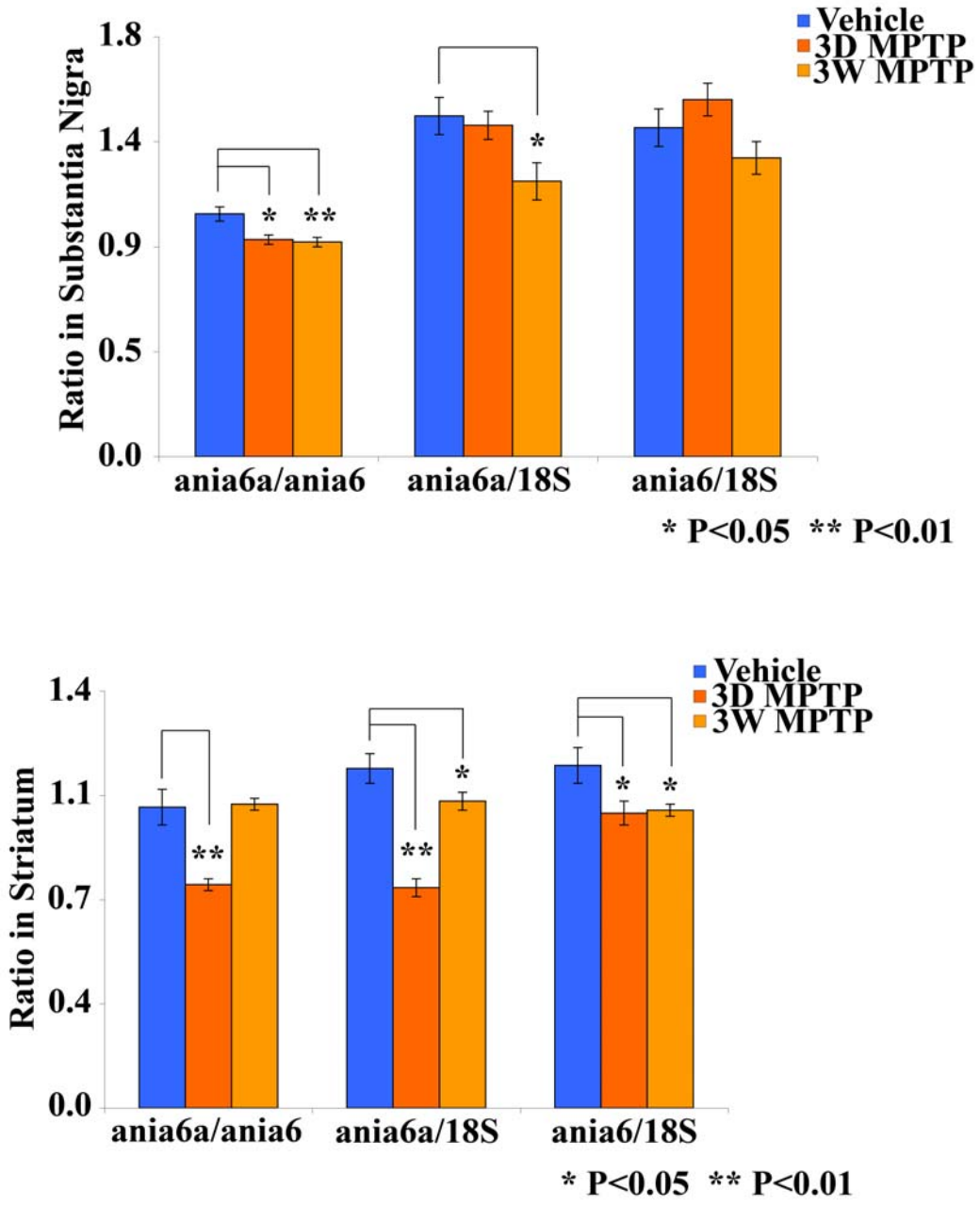


Fig. 6. Ania 6 splice variants in the brains of mice after chronic MPTP/P treatment. The mice were euthanized 3 days (D) or 3 weeks (WK) after toxin administration.

We also observed a significant decrease in the ratio of ania6a/ania6 in the striatum and substantia nigra of chronically MPTP-treated mice that were euthanized 3 days post-MPTP/probenecid treatment (Fig. 6). This decrease persisted for 3 weeks post-treatment

in the substantia nigra (Fig. 6). In addition the ania 6a/18S and ania6/18S was reduced in the striatum at 3 days and 3 weeks and the ania6a/18S was decreased in the substantia nigra at 3 days (Fig. 6).

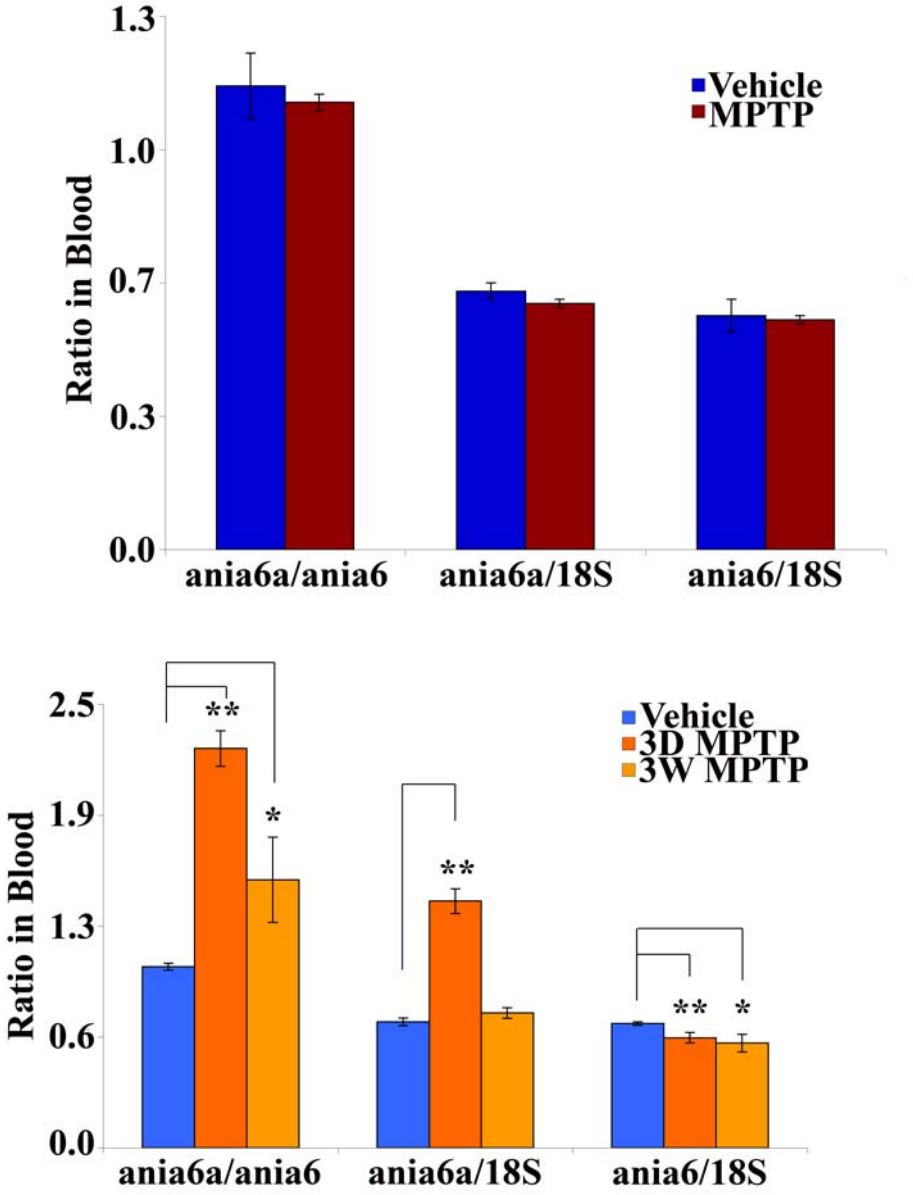
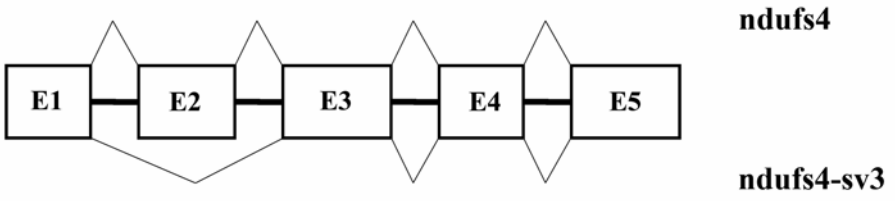


Fig. 7. Ania 6 splice variants in the blood of mice euthanized 3 days after acute MPTP treatment, or 3 days (D) or 3 weeks (WK) after chronic MPTP administration.

We also observed an increase in the ratio of ania6a/ania6 in the blood of chronically treated mice that persisted for 3 weeks post-MPTP/probenecid treatment, but no change was observed in acutely treated mice (Fig. 7). The increase in the chronically treated animal was a result of an increase in the ania6a/18S and a decrease in the ania6/18S ratio (Fig. 7). **These results from the ania6 studies indicate that some of the splice variant changes in the brain and blood persist for at least 3 months in the mouse model.**

Ndufs4 encodes ubiquinone oxidoreductase complex I, one of the structural subunits of the mitochondrial NADH, We examined two of the splice variants of the ndufs4 transcirpt, ndufs4 which includes exon2, and ndufs4-sv3, which skips exon 2 (Fig. 8, top panel). The results from RNA prepared from the striatum indicated that there is a decrease in the ndufs4-sv3/18S ratio in acutely treated animal (Fig. 8, middle panel) and an increase in the ndufs4-sv3/ ndufs4 ratio in the chronically treated mice (Fig. 8, bottom panel). We have not yet tested the ndufs4 splice variants present in the substantia nigra.



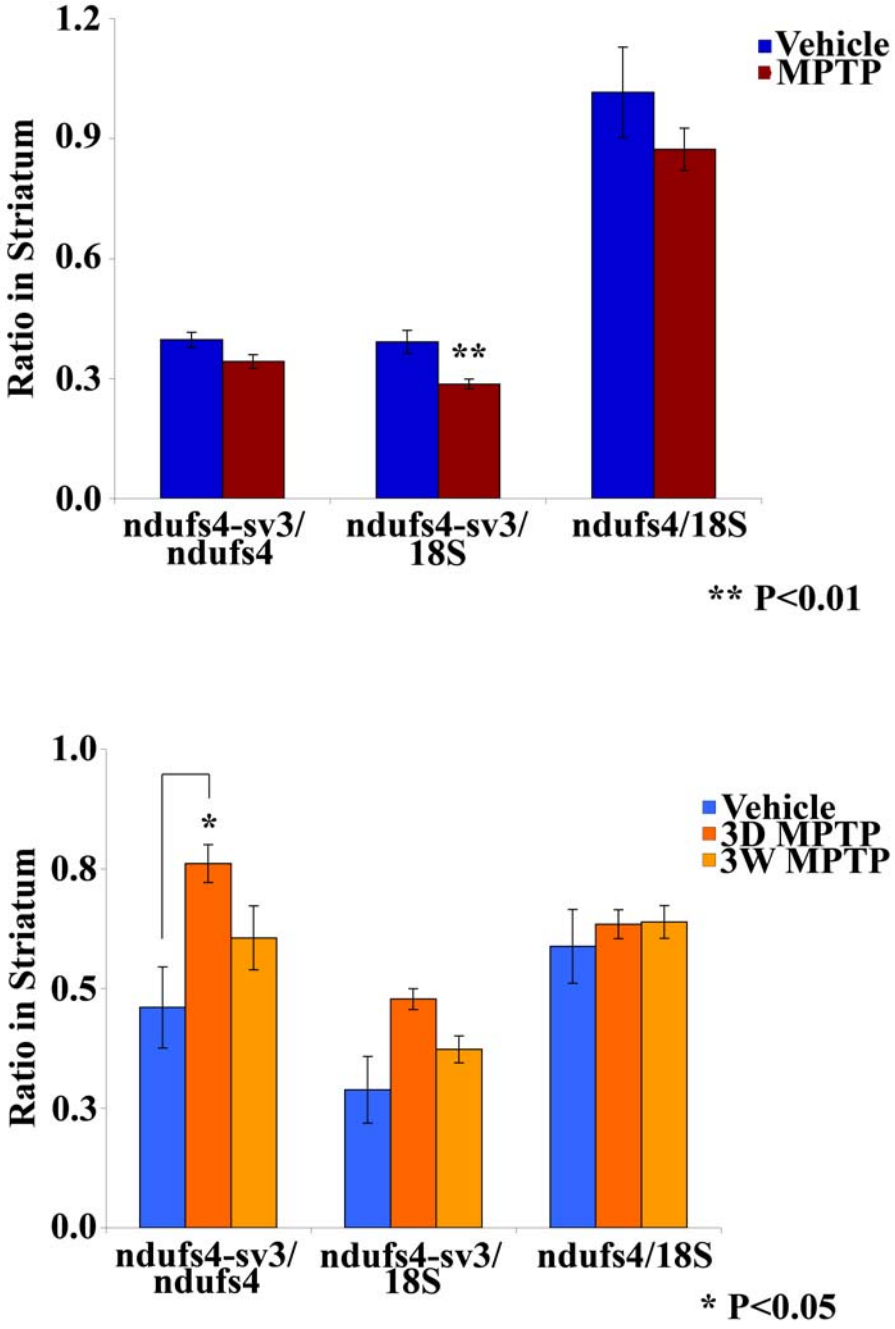
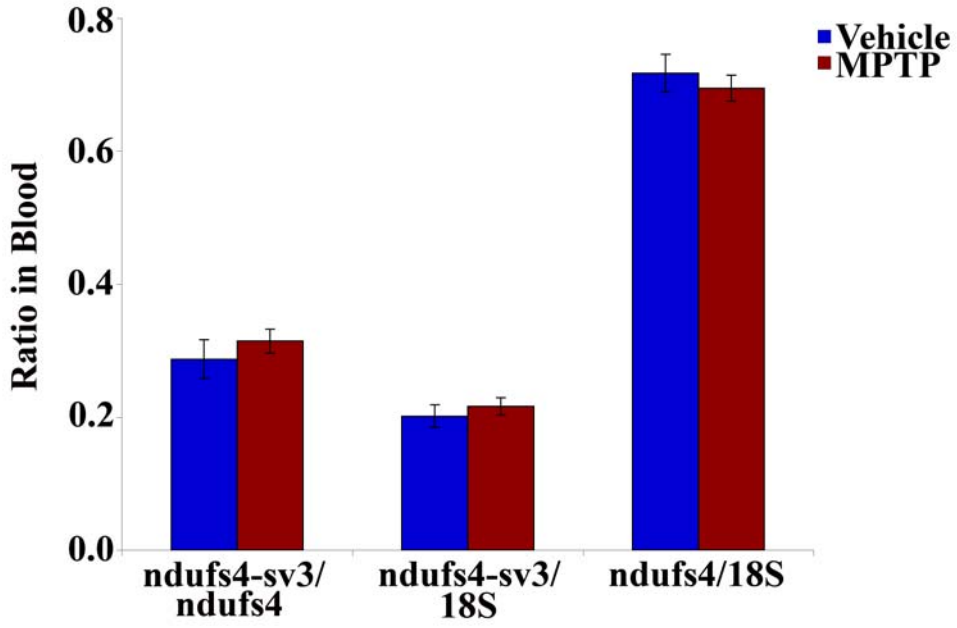


Fig. 8. Ndufs4 splice variants in the brains of mice euthanized 3 days after acute MPTP treatment (top panel), or 3 days (D) or 3 weeks (WK) after chronic MPTP administration (bottom panel).

In the blood we observed no changes in the ratio of the ndufs4 splice variants after acute treatment (Fig. 9, top panel). In the chronically treated mice we observed a decrease in the ndufs4-sv3/ ndufs4, ndufs4-sv3/18S and ndufs4/18S ratio at 3 days (Fig. 9, bottom panel). All the changes in ndufs4 splice variants persisted for 3 weeks except the ndufs/18S ratio. **The results from the ndufs4 splice variant studies suggest that there is a dysregulation of ndufs4 splicing after MPTP treatment.**



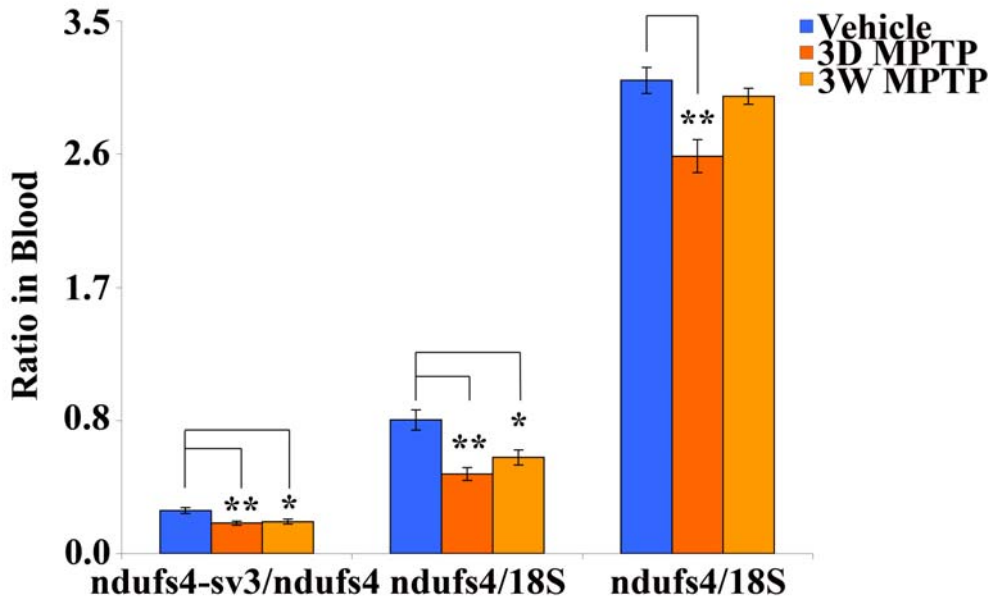


Fig. 9. Ndufs4 splice variants in the blood of mice euthanized 3 days (D) or 3 weeks (WK) after chronic MPTP administration.

Objective 2: To determine if the distribution of those splice variants found in rodent models of Parkinsonism correlate with regions in the brain that are affected in PD.

Quantitative *in situ* hybridization (ISH) has been done on the striatum and midbrains of mice treated chronically MPTP (see Fig. 4). In this work, we have begun studies of the splice variants of AChE and ANIA-6. There have been technical difficulties with these probes due to their short length. Nevertheless, we are trouble-shooting these problems. Figure 4 localizes the gene expression of one splice variant (AChE-R) in the midbrain of mice treated with saline or MPTP. We expect to complete this objective later in the year and well within the timeframe expressed in the Statement of Work (within 32 months from the start of the project).

Objective 3: To determine if the splice variants whose regulation is altered in rodent models are altered in the blood of PD patients compared to age-matched controls.

We have just received IRB approval from the USAMRMC (see attached) and from the two Universities with whom we collaborate. We are taking advantage of the availability of 2 well-characterized cohorts of PD patients, the first is at the University of Chicago Movement Disorders Center. These patients are registered by the co-Director, Un Kang, M.D. The second is at the Medical College of Wisconsin Movement Disorders Clinic with the director, Karen Blindauer, M.D. Blood will be collected at the time of the patient's initial neurological evaluation. These patients will be mostly drug naïve and in Stage I-II (Hoehn and Yahr scale) and will include males and females. We expect that most patients will be Medicare recipients and, thus, have equal access to health care. Control subjects (n = 25 and age-matched) will be spouses of the patients or obtained from other patient populations. Blood samples will be drawn via venipuncture and collected in heparin-coated tubes. An aliquot (100microl) will be placed in an RNase-free 1.5 ml microcentrifuge tube. All samples will be coded and immediately sent to the Chicago Medical School for RNA analysis.

Key Research Accomplishments summarized:

- Successfully extracted RNA from blood, brain (striatum and substantia nigra) and nasal epithelium of mice treated with MPTP, and quantified splice variants using TaqMan assays (Perkin-Elmer-Applied Biosystems)
- Successfully correlated changes in splice variant ratios with the loss of dopaminergic neurons from the substantia nigra, poor performance on behavioral tests and dopamine levels in the striatum.
- In the blood, the ratio of fosB/18S rRNA and rgs9-2/18S rRNA transcripts decreased after acute MPTP treatment and the fosB/18S rRNA and rgs9-2/rgs9-1 transcript ratios decreased after chronic MPTP/probenecid treatment.
- In the blood, there is a significant increase in the ratio of AChE-R/AChE-S after acute MPTP treatment but a decrease 3 days after chronic treatment.
- Gene Expression studies are underway but there are, as yet, no quantifiable results.

- IRB approvals for studies of human blood are in place.

Reportable Outcomes

Published or submitted for publication the following:

- Potashkin JA, Kang UJ, Loomis PA, Jodelka FM, Ding Y, Meredith GE (2007) MPTP administration in mice changes the ratio of spice isoforms of fosB and rgs9. Brain Res, *in press*.
- Meredith, GE, Totterdell S, Potashkin JA, Surmeier DS (2007) Modeling PD pathogenesis in mice: Advantages of a chronic MPTP protocol. Parkinsonism Relat Disord, *in press.*
- Potashkin JA, Loomis PA, Jodelka FM, Meredith GE (2007) FosB and rgs9 splic variants are biomarkers for MPTP exposure. Neuroscience, submitted.
- Potashkin JA, Kang UJ, Loomis PA, Ding Y, Jodelka FM, Meredith GE (2007) Dysregulation of AChE splicing in acute and chronic models of Parkinson's disease. Eukaryotic RNA processing, Cold Spring Harbor, NY.
- Potashkin JA, Kang UJ, Loomis PA, Ding Y, Jodelka FM, Pitner J, Meredith GE (2007) Dysregulation of AChE splicing in acute and chronic models of Parkinson's disease. Soc Neurosci Abst., *in press*

Conclusion

The second year of this grant has seen the completion of fosB, rgs9, ache, ania6 and ndufs4 splicing studies in the blood of the MPTP mouse model. We still need to examine the splicing of ndufs4 in the substantia nigra after MPTP treatment. We have started studies of the gene expression of different variants with the goal of localizing changes in the brain. We have found a dysregulation of splicing for all 5 pre-mRNAs after MPTP treatment in mice. These results indicate that splice variants of transcripts in the blood could help identify acute and long-term exposure to toxins that facilitate the progression of Parkinsonism in humans. We hope to identify the presence of fosB, rgs9, ache, ania6 and ndufs4 splice variants in the blood of newly diagnosed human PD patients during the third year of this study in order to establish these transcripts as biomarkers of disease.

Progress this year includes 2 abstracts, two peer-reviewed publications in press and one submitted manuscript. IRB approval for the human studies is now in place and those studies will begin shortly.

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Appendices

Appendix I. Potashkin JA, Kang UJ, Loomis PA, Jodelka FM, Ding Y, Meredith GE (2007) MPTP administration in mice changes the ratio of spice isoforms of fosB and rgs9. Brain Res, *in press*.

Appendix II. Potashkin JA, Loomis PA, Jodelka FM, Meredith GE (2007) FosB and rgs9 splice variants are biomarkers for MPTP exposure. Neuroscience, submitted.

Appendix III. Initial Approval for the Protocol, “Identification of Splice Variants as Molecular Markers in Parkinson’s Disease,” Submitted by Gloria E. Meredith, PhD, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois, Proposal Log Number 05090011, Award Number W81XWH-05-1-0580, HRPO Log Number A-13416.

MPTP administration in mice changes the ratio of splice isoforms of fosB and rgs9

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Number of total pages: 24
Number of text pages: 20
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Number of tables: 2

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Abstract

Most cases of Parkinson's disease (PD) are sporadic, suggesting an environmental influence on individuals affected by this neurodegenerative disorder. Environmental stresses often lead to changes in the regulation of splicing of pre-mRNA transcripts and this may lead to the pathogenesis of the disease. A 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/probenecid mouse model was used to examine the changes in the splicing of the fosB and rgs9 transcripts. The ratio of ΔfosB/fosB transcript was decreased in the substantia nigra and unchanged in the striatum after acute MPTP treatment. The ΔfosB/fosB transcript ratio decreased initially and then increased in the striatum of chronically MPTP-treated animals, due to different degrees of reduction for the splice variants over time, whereas the ratio was unchanged in the substantia nigra. The ratio of rgs9-2/rgs9-1 transcript decreased in the substantia nigra of mice after acute MPTP-treatment and increased temporarily in the striatum after chronic MPTP-treatment. There was an increase in the ΔFosB/FosB and RGS9-2/RGS9-1 protein ratios three weeks and three days post-treatment, respectively, in chronically-treated mice. The data indicate that the pattern of splice-isoforms of fosB and rgs9 reflects the brain's immediate and long-term responses to the physiological stress associated with Parkinsonism.

Classification terms:

Section: 1. Cellular and Molecular Biology of Nervous Systems

Keywords: pre-mRNA splicing, dopamine, MPTP, FosB, RGS9, neurodegeneration

1. **Introduction**

Alternative splicing is responsible for producing several mRNAs from a single transcript. While this process could be viewed as beneficial because it is a strategy for adaptation, it can also become detrimental when translation of particular splice variants results in pathogenic changes (Potashkin and Meredith, 2006; Stamm et al., 2005). Regulatory splicing factors affect the expression of multiple pre-mRNAs, often functioning to coordinate changes in protein isoform expression and function. Even though many modifications derived from alternative splicing are subtle, they can influence important signaling features of the nervous system, such as ion channel properties, half-life of proteins, and loss or gain in receptor function (Stamm et al., 2005). Numerous endogenous and environmental stimuli can alter splicing and moreover errors in splicing are associated with a wide variety of disorders including cancer, Alzheimer's disease and Parkinson's disease (PD) (Potashkin and Meredith, 2006; Stoilov et al., 2002).

Postmortem analysis of the brains of PD patients found that ΔFosB and RGS9 proteins, both products of alternative splicing and involved in various aspects of dopamine signaling (Cenci et al., 1999; Doucet et al., 1996; Gold et al., 1997; Hope et al., 1992; Perez-Otano et al., 1998; Rahman et al., 1999; Rahman et al., 2003), were expressed at higher levels within the striatum when compared to aged matched controls (Tekumalla et al., 2001). ΔFosB was also elevated in the striatum of experimental models of PD and L-DOPA-induced dyskinesia (Cenci, 2002; Pavon et al., 2006; Perez-Otano et al., 1998). Relative to the transcription factor FosB, ΔFosB is a more stable protein and thus accumulates with time in response to chronic stimuli (McClung et al.,

2004; Nestler, 2001). Since ΔFosB may compete with CREB binding at CRE sites, accumulation of this splice product could inhibit CREB transcription (McClung et al., 2004). Together, these results suggest that the enhancement of the ΔFosB/FosB protein ratio can be a long-term mechanism for altering transcription dynamics. RGS9, is among the many regulators of G proteins and acts by accelerating the termination of effector stimulation, thus, suppressing signaling efficacy of G-protein-coupled receptors (Dohlman and Thorner, 1997). The alternatively spliced product, RGS9-2, is especially enriched in the striatum but is also present in the substantia nigra (Kim et al., 2005). Work by Rahman and colleagues (Rahman et al., 1999) has shown that RGS9-2 dampens the $G_{i/o}$-coupled μ opioid receptor response (Rahman et al., 1999). RGS9-1, a second product of *rgs9*, is abundantly expressed in the retina and is thought to function as a GTPase-activating protein for transducin (Chen et al., 2000; He et al., 1998). Although the two isoforms share functional domains, the individual carboxy-terminal regions generated by alternative splicing greatly influence their sub-cellular localization. Retinal RGS9-1 is mainly localized to rod outer segment membrane while brain-specific RGS9-2 is predominately localized to neuronal nuclei (Bouhamdan et al., 2004; He et al., 1998). As yet unknown are the repercussions of the altered expression of these two splice variants of GTPase-accelerating proteins on the pathophysiology of Parkinson's disease.

MPTP has been used in primates, felines and rodents as a model for parkinsonism (reviewed in Smeyne and Goldowitz, 1989). Only some strains of mice are responsive to the toxin (Hamre et al., 1999; Riachi and Harik, 1988; Sundstrom et al., 1987). Acute administration of the neurotoxin MPTP to mice rapidly destroys many dopaminergic neurons of the nigrostriatal pathway and produces a Parkinsonian syndrome that mimics

some features of the motor disability in PD (Meredith and Kang, 2006; Przedborski and Vila, 2003). Chronic administration of this toxin, when combined with probenecid, also reduces nigrostriatal function, and depletes the pathway to a greater extent than other MPTP protocols (Petroske et al., 2001). In this study, we utilized both the acute and chronic protocols to characterize the regulation of splicing of fosB and rgs9 pre-mRNAs and examined how the splicing events are related to the loss of dopamine and the motor deficit. We focused our studies on the splice variants in the substantia nigra, where the dopamine neurons are particularly vulnerable to the toxin, and the striatum, where earlier studies indicate that the expression of these protein isoforms is altered in PD models (Cenci, 2002; Pavon et al., 2006; Perez-Otano et al., 1998). Understanding how and when these factors are alternatively spliced in Parkinsonian mice may reveal some of the adaptive mechanisms the nigrostriatal pathway uses to cope with degeneration resulting from oxidative stress (Meredith et al., 2004).

2. Results

Mice treated chronically with MPTP and probenecid revealed a significant decrease in dopamine in the striatum that was coincident with pronounced cell death of TH-immunopositive neurons in the substantia nigra pars compacta at three days and three weeks post-treatment (Table 1). This decrease in dopamine levels and the loss of dopaminergic neurons was correlated with a significant decrease in motor performance on the grid test for MPTP-treated mice when compared to vehicle treated controls (Table 1).

In order to determine if the splicing of fosB and rgs9 is altered in the development of Parkinsonism in mice after administration of MPTP we analyzed splice variants of both transcripts after acute and chronic MPTP treatment by semi-quantitative PCR. The gene for the transcription factor FosB produces two mRNAs by intron retention or splicing that is partially regulated by the splicing factor polypyrimidine tract binding protein (Fig. 1A, Marinescu et al., in press). The intron-retained transcript encodes full length FosB protein, and the intron-spliced transcript produces ΔFosB. There was a significant decrease in the ratio of ΔfosB/fosB in the substantia nigra (Fig. 1C and 1D), but no change in the striatum (Fig. 1B and 1D) after acute MPTP treatment. There was, however, a decrease in the fosB/18S rRNA in the striatum after acute treatment (Fig. 1B and 1D). After chronic MPTP/probenecid administration, there was a significant decrease in the ratio of ΔfosB/fosB in the striatum compared to vehicle-treated controls at three days, due mainly to a large decrease in ΔfosB (Fig. 2A and C). The ratio of ΔfosB/fosB mRNA in the striatum increased significantly at three weeks after chronic MPTP/probenecid administration (Fig. 2A). This was due to the increase of ΔfosB/18S rRNA and the further decrease of fosB/18S between three days and three weeks. There was no change in the ratio of ΔfosB/fosB in the substantia nigra after chronic MPTP/probenecid administration, but a decrease in the amount of fosB was observed at both three days and three weeks (Fig. 2B and C).

The rgs9 pre-mRNA has two splice variants that produce rgs9-2 and rgs9-1 (Zhang et al., 1996). Both transcripts of rgs9 contain the constitutively spliced exons 1-16 (Fig. 3A). The rgs9-1 mRNA also contains the entire exon 17 (A and B), whereas rgs9-2 contains exon 17A and exons 18 and 19 due to the use of an internal splice donor

site (Fig. 3A). Following acute MPTP treatment, there was a significant decrease in the ratio of rgs9-2/rgs9-1 in the substantia nigra compared to vehicle-treated controls (Fig. 3C and D), but no change in the striatum (Fig. 3B and D). In addition, three days after chronic, MPTP/probenecid treatment, there was a significant increase in the ratio of rgs9-2/rgs9-1 in the striatum compared to vehicle-treated controls (Fig. 4A and C), caused mainly by a large decrease in the rgs9-1/18S ratio three days post-treatment. This effect was not evident in mice euthanized 3 weeks post-treatment. No change in the ratio of rgs9-2/rgs9-1 was detected in the substantia nigra after chronic MPTP/probenecid treatment, however, we noted a decrease in the rgs9-2/18S three days post-treatment (Fig. 4B and C).

In order to determine if the changes in splicing ratios were reflected in protein levels, we used Western blot analysis to examine ΔFosB, FosB, RGS9-1 and RGS9-2 within the striatum of both chronically vehicle- and MPTP/probenecid-treated mice. The results indicated an increase in the ΔFosB/FosB protein ratio both three days (Fig. 5B and C) and three weeks post-MPTP treatment (Fig. 5A and C). The amount of both ΔFosB and FosB normalized to β-Tubulin protein increased three days post treatment (Fig. 5B and C). There was also an increase in the RGS9-2/RGS9-1 ratio three days post-treatment that did not persist for three weeks (Fig. 6A and C). The amount of both RGS9-1 and RGS9-2 increased three days post treatment when normalized to β-Tubulin protein, similar to the FosB proteins (Fig. 6B and C).

3. Discussion

One of the most widely used models for Parkinson's disease is produced by administering MPTP to mice. Exposure to this toxin is often fatal to dopaminergic neurons in the substantia nigra which in turn results in substantially reduced dopamine levels within the striatum. We have used a chronic and acute MPTP model to test whether exposure to toxin is reflected in changes in the splicing of pre-mRNA within the brain. The results revealed that in the striatum, the abundance of splice variants of fosB and rgs9 is altered in mice treated chronically with MPTP and probenecid, but unchanged 72 hours after acute MPTP treatment. This dysregulation of striatal splicing in the chronic model correlates well with the loss of dopamine in the striatum and the loss of agility in the grid test. In the substantia nigra, the ratio of rgs9-2/ rgs9-1 and ΔfosB/fosB, is altered after acute, but not chronic, MPTP administration, indicating that a shift in rgs9 and fosB splicing is particularly sensitive to the rapid loss of dopaminergic neurons following toxin exposure (Jackson-Lewis et al., 1995, present results).

An acute response to changes in dopamine receptor stimulation is the induction of immediate early gene (IEG) expression. One such IEG encodes the transcription factor FosB. Loss of dopaminergic neurons in animal models of Parkinson's disease induces expression of the truncated form of FosB, ΔFosB (Cenci et al., 1999; Doucet et al., 1996; Hope et al., 1994; Perez-Otano et al., 1998). Additionally, dopamine agonists stimulate supersensitive dopaminergic receptors in animals that have lost their dopamine supply to the striatum, and presumably induce even higher levels of ΔFosB (Hope et al., 1994). The consequences of the elevated ΔFosB expression are unclear, although ΔFosB most likely alters gene expression with downstream affects on behavior (McClung and Nestler, 2003).

The largest change seen in our study was a decrease in both ΔfosB and fosB mRNA in the striatum after chronic MPTP/probenecid administration. In contrast to the chronic treatment, there was no change in the amounts of ΔfosB or fosB mRNAs in the striatum after acute MPTP administration. Coincident with the change after chronic treatment was a significant decrease in the ratio of ΔfosB/fosB mRNA as compared to vehicle control levels in the striatum three days post-treatment. Interestingly, when examined three weeks post-treatment, the ratio of ΔfosB/fosB mRNA was significantly increased as compared to control levels. This change in the ratio was due to an increase in the amount of ΔfosB mRNA and a further decrease in the amount of fosB mRNA compared to the three day post- chronic treatment values. By comparison, chronic administration of amphetamine, which augments rather than decreases striatal dopamine levels, did not produce a significant change *in vivo* in the ratio of ΔfosB to fosB splice variants in the striatum, whereas acute amphetamine or stress increase the expression of both fosB and ΔfosB in the striatum (Alibhai et al., 2007). These results along with those reported in this study suggest that it is the nature of the toxin or drug that produces the unique molecular signature of splice variants in the brain, not simply the chronic or acute nature of the treatment.

The changes we observed in mRNA splice variant ratio of fosB were reflected in the ratio of the protein products after chronic MPTP/probenecid treatment. The changes in the amount of mRNA of each fosB splice variant normalized to 18S RNA, however, did not reflect the protein products in the striatum. In the striatum we observed a significant increase in ΔFosB and FosB in the MPTP/probenecid chronically treated animals that were euthanized three days post-treatment. The discrepancy between

changes in RNA and protein concentrations exists because of the many regulatory steps in gene expression between transcription and protein production including pre-mRNA splicing, RNA stability, RNA transport, translation and protein modification. For example, at least three factors have been identified that contribute to ΔFosB protein accumulation upon exposure to addictive drugs or during times of stress. These include activation of the *fosB* gene and induction of ΔfosB mRNA (Chen et al., 1995); the absence of a C-terminal destabilizing domain of ΔFosB protein (Acquaviva et al., 2001); and phosphorylation of an serine residue on ΔFosB, which inhibits proteosomal degradation (Ulery et al., 2006). The impact of changes in fosB RNA stability, RNA transport and translation remain unknown.

The regulation and role of the RGS9 proteins are intimately intertwined with G-protein coupled receptor signaling (Dohlman and Thorner, 1997). Many of the motor deficits associated with PD are associated with changes in signaling at the D2 receptor (Alexander and Crutcher, 1990; Alexander et al., 1990; Gerfen, 2000a; Gerfen, 2000b), which is G-protein coupled and may be selectively regulated in the striatum by RGS9-2 (Rahman et al., 2003). In the current study, the increase in rgs9-2/rgs9-1 three days after chronic MPTP/probenecid treatment in the striatum, is reflected in the increase in RGS9-2 and RGS9-1 proteins. RGS9-2 reduces the efficacy of D2 receptor signaling (Rahman et al., 1999). Therefore, the change in the amount of RGS9-2 and RGS9-1 may produce an imbalance in dopamine signaling that may be partially responsible for, or a compensatory response to, disease progression.

G-protein-gated inward rectifier K+ (GIRK) channels respond to Gi activation and RGS9-2 accelerates D2-induced GIRK currents (Rahman et al., 2003). The *weaver*

mutation affects a GIRK2 channel gene (Patil et al., 1995), which appears to contribute to activity-dependent cell death of dopaminergic neurons (Slesinger et al., 1996). In addition to a loss of the GIRK2-mediated hyperpolarizing current (Surmeier et al., 1996; Lauritzen et al., 1997), there is evidence that the functional consequences of this mutation can lead to a reduction of channel selectivity for K+ and a gain of constitutive activity, allowing Na+ and Ca2+ ions to permeate into cells in a G-protein-independent manner (Kofuji et al., 1996; Navarro et al., 1996; Silverman et al., 1996; Slesinger et al., 1996; Tong et al., 1996). Such changes could contribute to the activity-dependent degenerative process of dopaminergic neurons and striatal dopamine innervation, along with a decrease in striatal dopamine levels (Schmidt et al., 1982; Roffler-Tarlov et al., 1984; Triarhou et al., 1988) that typically take place in the wv/wv mice (Roffler-Tarlov et al., 1984; Triarhou et al., 1988; Bayer et al., 1995; Verney et al., 1995; Broome et al., 1999). Thus, dopaminergic cell death observed in animal models of PD with neurotoxins (i.e., MPTP) as well as spontaneously in inbred strains of mice, such as the *weaver* (wv/wv) mutant mice (Bayer et al., 1995; Lauritzen et al., 1997) may be due to changes in GIRK currents resulting from mutations in these channel genes or, perhaps, alterations in the abundance of RGS9 proteins, as in the present results.

In human PD, an increase in striatal expression of ΔFosB and RGS9-2 was observed in post-mortem brains of PD patients (Tekumalla et al., 2001). These were patients who presumably had been on medication for a number of years thereby raising the possibility that dopaminergic medications contributed to these reported alterations. The present study suggests that changes in FosB and RGS9 protein isoform expression occur even without medications, although medications may further alter the isoform

expression. Still unknown at this time is whether these changes are due to the toxic insult itself or a regional adaptation in the brain to the toxin.

Recently, Chisa and Burke (Chisa and Burke, 2007) showed splice isoform ratios are one of the most invariant phenotypes that may be measured in a mammalian population. The splice isoform ratios were robust biomarkers, indicating that they showed little inter-individual variability with genetic diversity. In addition, contrary to other markers that show an increase in variability in elderly populations, the splice variant ratios showed a tight distribution independent of the age of the animal. Therefore, the ratio of splice variants may be beneficial as biomarkers that reflect the physiological state of cells and tissues. It also suggests that changes in the ratio of splice variants may reflect negative consequences for the animal. In humans, a 5% changes in splice isoforms ratio may be linked to adult onset diseases (Bertram et al., 2005; Gretarsdottir et al., 2003; Ueda et al., 2003). Thus the changes in ratio of ΔfosB/fosB and rgs9-2/rgs9-1 in the striatum that we observe after chronic MPTP treatment may reflect an adaptation to disease progression. Further studies will be needed to determine whether splicing plays a direct role in the development of Parkinson's disease.

4. Experimental Procedures

Animals. One hundred, adult, C57BL/6 male mice (Charles River Laboratories, Wilmington, MA, USA) weighing 22-24 g were housed two-four per cage with food and water available *ad libitum*. Experiments were conducted in accordance with the U.S. Public Health Service Policy and the National Institutes of Health Guide on the Humane Care and Use of Laboratory Animals. All animal treatments including anesthesia were

carried out following protocols approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee

The acute protocol involved 4 injections of 20 mg/kg MPTP (in saline), every 2 hours. Mice were euthanized 3 days later. Another group of mice were treated chronically as previously described (Petroske et al., 2001). Briefly, mice were injected twice each week, at 3.5-day intervals, for 5 weeks with MPTP (25 mg/kg in saline, s.c) and probenecid (250 mg/kg, i.p.). Controls were injected with saline as vehicle. All mice were then euthanized either at 3 days or 3 weeks after this treatment. Mice for splicing studies and HPLC measurements of striatal dopamine were euthanized by cervical dislocation followed by decapitation and their brains dissected over ice.

Tyrosine hydroxylase immunohistochemistry. Dissected midbrains were placed in 3% paraformaldehyde in 0.1M phosphate buffer for seven days at 4 ^{o}C. Midbrains were blocked and sunk in 20% sucrose. Coronal sections were cut on a cryostat at 50 µm, and after a random start, every fourth section through the SN was collected in series. Sections were immunoreacted with a monoclonal mouse anti-tyrosine hydroxylase (TH; Diasorin, Stillwater, MN) and using the reagents in a special kit to reduce background on mouse tissue (Mouse-on-Mouse Kit; Vector Laboratories, Burlingame, CA). Sections were incubated for 24 hours at 4^{o}C in anti-TH sera diluted 1:2000, followed by a two hour incubation at room temperature (RT) in biotinylated anti-mouse IgG (1:300, Vector Laboratories), and then a 45 min incubation (RT) in avidin-biotin complex (Elite kit, Vector Laboratories). Sections were reacted in (3,3'– diaminobenzidine hydrochlolride (DAB) and 0.01% H_2O_2, mounted onto slides, counterstained with cresyl violet (Nissl),

dehydrated and topped with coverslips. The total number of TH-positive, Nissl-stained neurons was estimated with optical dissectors in a known fraction of the volume that had been optimized in a pilot study (StereoInvestigator, Microbrightfield, Williston, VT; Dervan et al., 2005). This was accomplished by superimposing a counting frame on a high-magnification image of the region, using a high numerical aperture (1.35, oil immersion objective) to sample cells in a systematic random manner. The sampling scheme was evaluated by coefficients of error (CE). Reference volumes were established using Cavalieri principles. Groups were compared with standard statistics (Student's t-test).

Behavioral testing. All mice treated chronically with MPTP/probenecid were analyzed with a behavioral test carried out the day before they were euthanized. Normally, any behavioral test carried out close to the time of toxin treatments is not valid since MPTP creates a peripheral toxicity and it becomes difficult to assess whether the motor deficit is due to the toxicity or the actual loss of dopaminergic neurons [24]. We therefore did not test mice treated acutely with MPTP. We did test the mice that were treated chronically with MPTP at 3 days and 3 weeks so that a comparison could be made between the two time points. The impact of the toxin or vehicle was assessed using a novel grid test that provides a measure of coordination and balance on a mesh grid (Meredith and Kang, 2006). The grid is constructed of a horizontal square (15 cm^2) of metal mesh attached with staples to 4 cardboard walls. The openings in the mesh are 0.5 cm^2. The mouse is lifted by the tail and placed in the center of the grid, which is then rotated 180^o suspending the mouse approximately 20 cm above the floor. The mouse is allowed to move freely while upside down and is videotaped for 60 seconds. Each unsuccessful step is scored as a fault, and a

ratio is established: total foot faults/total steps. Animals must take a minimum of 10 total steps to be included in the data set and must stay on the grid at least 30 sec. Each mouse is tested during three trials on a single day with an extended rest period of at least 45 mins between trials. The mice were coded and the individual analyzing the videotapes is blind to the treatment groups. Data for each group was pooled and groups were compared with a Student's t-test.

HPLC Assessment of Dopamine and Metabolites in Brain Tissue. HPLC determinations of striatal dopamine were carried out using dissected striata tissue suspended in 0.4 ml of 0.1 M perchloric acid as previously described (Chen et al., 2005). Briefly, samples were homogenized for 20 sec, sonicated for 20-30 sec on ice and centrifuged at 2,300 X g for 5 min at 4°C. The supernatant was filtered through a YM-10 Microcon filter (Millipore, Billerica, MA) and frozen at -80°C. Dopamine levels in aliquots of the samples were detected by HPLC (Shimadzu LV-10AD, Shimadzu Scientific Instruments, Inc., Columbia, MD) in a mobile phase consisting of 36 mM sodium acetate, 150 μM sodium octyl sulfate, 100 μM EDTA and 5% (v/v) CH_3CN, pH 3.74. The samples were chromatographed by a Primesphere column (C18-HC, 250x4.60 mm, 5 micron, Phenomenex, Torrance, CA) at a constant flow rate of 1ml/min; the concentration of dopamine was then determined by electrochemical detection (Coulochem II, ESA, Chelmsford, MA). Results are expressed as dopamine/protein (pg/μg).

RNA Preparation and Amplification by Polymerase Chain Reaction. The striatum and substantia nigra were removed from the brains of 11-16 mice per group and flash frozen

in liquid nitrogen. Total RNA was extracted from brain tissue using the SV total RNA isolation system as per the manufacturer's instructions (Promega, Madison, WI). RNA was eluted in 100 μl of nuclease-free water. The RNA was aliquoted and stored at -80 °C. cDNA was generated from the RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following instructions provided by the manufacturer. The PCR reactions were denatured at 95 °C for 2 min; followed by 18-35 cycles of 20 sec at 95 °C, 30 sec at 54°C (for rgs9) or 57°C (for fosB), 30 sec at 72 °C and a final extension for 3 min at 72 °C. The PCR reactions for 18s were denatured at 95 °C for 2 min; followed by 18 cycles of 30 sec at 95 °C, 30 sec at 58°C, and 30 sec at 72 °C. After amplification, PCR products were separated on agarose gels containing 5 μg of ethidium bromide (10 mg/ml stock). The intensity of each band was quantified using the Kodak ID Image Analysis Software (Kodak, Rochester, NY). Quantification of serial dilutions of the PCR products indicated the density measure was within linear range. Microsoft Excel 2004 for Mac version 11.0 software (Bellevue, WA) was used to make statistical comparisons among groups using a t-test analysis for two samples assuming unequal variance. Significance was set at $p < 0.05$.

Protein Analysis by Western blotting. Protein was extracted from brain tissue in ice-cold Homogenization Protein Buffer containing 20 mM Tris-HCl (pH 7.4), 10% sucrose, 1 mM EDTA, 0.1% SDS and a Protease Inhibitor Cocktail tablet (Roche Diagnostics, Mannheim, Germany). Each sample was sonicated for 16 pulses and then centrifugation at 11,000 x g for 5 min at 4°C. Protein concentration was determined using the BCA Protein assay (Pierce, Rockford, IL) according to manufacturer's instructions. An equal

amount of 2X SDS sample buffer (125 mM TrisHCl pH 6.8, 20% glycerol, 4% SDS, 0.2% 2-mercaptoethanol (2-ME) and 0.001% bromphenol blue) was added to the samples and then boiled for 5 min and resolved on 4-15% polyacrylamide gradient gels (25 μg protein/lane). Proteins were transferred onto PVDF transfer membranes (Fisher Scientific, PA) for immunoblotting using a FosB, RGS9 or β-tubulin antibody (Santa Cruz, CA). The ECL method (Amersham Biosciences, Buckinghamshire, UK) was used to detect bound antibody. The optical density of each band was determined using Kodak 1D Image Analysis Software (Kodak).

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Figure Legends

Fig. 1. In vivo expression of fosB splice isoforms in the striatum and substantia nigra after acute MPTP treatment. Alternative splicing of fosB pre-mRNA produces a fosB and fosB mRNA by the splicing or retention, respectively, of intron 4 (A). Mice were treated acutely with MPTP and RNA from the striatum (B) or substantia nigra (C) was analyzed 3 days after the last injection. A representative gel showing the products of the PCR reactions (D). Data are expressed as mean±sem (n=3 acute saline substantia nigra, 10 acute saline striatum, 6 acute MPTP substantia nigra, 23 acute MPTP striatum), *p<0.01, #p<0.001.

Fig. 2. In vivo expression of fosB splice isoforms in the striatum and substantia nigra after chronic MPTP treatment. Mice were treated chronically with MPTP and RNA from the striatum (A) or substantia nigra (B) was analyzed 3 days (D) or 3 weeks (W) after the last injection. A representative gel showing the products of the PCR reactions (C). Data are expressed as mean±sem (n=5 chronic saline substantia nigra, 11 chronic saline striatum, 7 chronic MPTP 3D substantia nigra, 8 chronic MPTP 3W substantia nigra, 15 chronic MPTP 3D striatum, 15 chronic MPTP 3W striatum), **p<0.001.

Fig. 3. In vivo expression of rgs9 splice isoforms in the striatum and substantia nigra after acute MPTP treatment. Alternative splicing of rgs9 pre-mRNA produces an rgs9-1 and rgs9-2 mRNA by the inclusion of the entire exon17 or exon17A, exon18 and exon19, respectively (A). Mice were treated acutely with MPTP and RNA from the striatum (B)

or substantia nigra (C) was analyzed 3 days after the last injection. A representative gel showing the products of the PCR reactions (D). Data are expressed as mean±sem (n=7 acute saline substantia nigra, 7 acute saline striatum, 7 acute MPTP substnatia nigra, 15 acute MPTP striatum), *p<0.05.

Fig. 4. In vivo expression of rgs9 splice isoforms in the striatum and substantia nigra after chronic MPTP treatment. Mice were treated chronically with MPTP and RNA from the striatum (A) or substantia nigra (B) was analyzed 3 days (D) or 3 weeks (W) after the last injection. A representative gel showing the products of the PCR reactions (C). Data are expressed as mean±sem (n=5 chronic saline substantia nigra, 11 chronic saline striatum, 7 chronic MPTP 3D substantia nigra, 8 chronic MPTP 3W substantia nigra, 15 chronic MPTP 3D striatum, 15 chronic MPTP 3W striatum), *p<0.01, ** p<0.001.

Fig. 5. Analysis of FosB protein in the striatum after chronic MPTP treatment. Mice were treated chronically with MPTP/probenecid and protein from the striatum was analyzed 3 days (D) or 3 weeks (W) after the last injection. Proteins were analyzed by Western blot assay using a FosB (A,B and C), or □-tubulin antibody (B and C). A representative gel showing the products of the PCR reactions (C). Data are expressed as mean±sem (n=4-7 per group), *p<0.05, **p<0.01.

Fig. 6. Analysis of RGS9 protein in the striatum after chronic MPTP treatment. Mice were treated chronically with MPTP/probenecid and protein from the striatum was

analyzed 3 days (D) or 3 weeks (W) after the last injection. Proteins were analyzed by Western blot assay using a RGS9 (A, B and C) or □-tubulin (B, C) antibody. A representative gel showing the products of the PCR reactions (C). Data are expressed as mean±sem (n=4-7 per group), *$p<0.05$.

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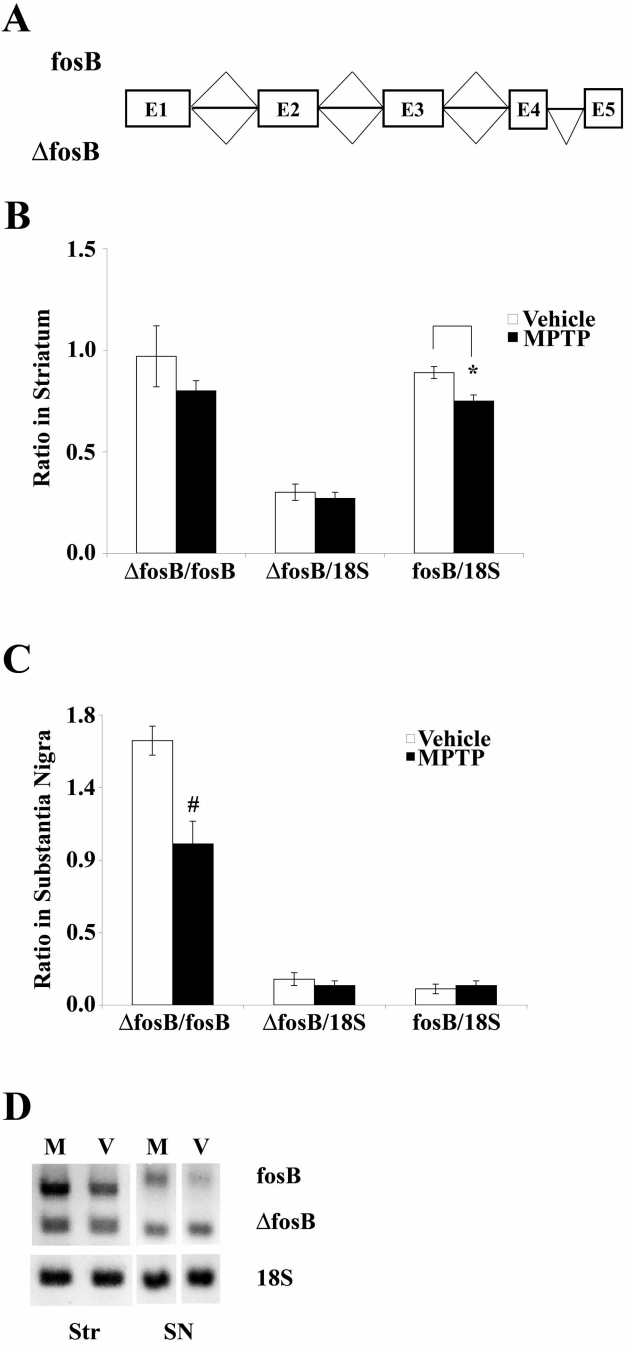
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fosB
ΔfosB
E1
E2
E3
E4
E5
B
Ratio in Striatum
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1.0
0.5
0.0
Vehicle
MPTP
*
ΔfosB/fosB
ΔfosB/18S
fosB/18S
C
Ratio in Substantia Nigra
1.8
1.4
0.9
0.5
0.0
Vehicle
MPTP
#
ΔfosB/fosB
ΔfosB/18S
fosB/18S
D
M
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V
fosB
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Str
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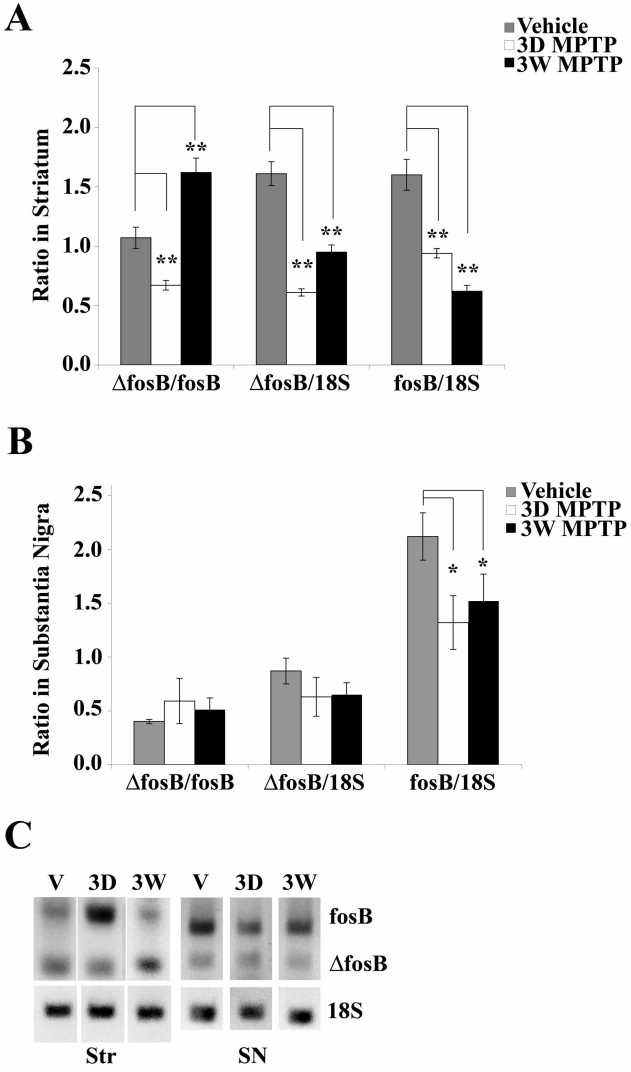
A
Vehicle
3D MPTP
3W MPTP
Ratio in Striatum
2.5
2.0
1.5
1.0
0.5
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**
ΔfosB/fosB
ΔfosB/18S
fosB/18S
B
Ratio in Substantia Nigra
*
C
V
3D
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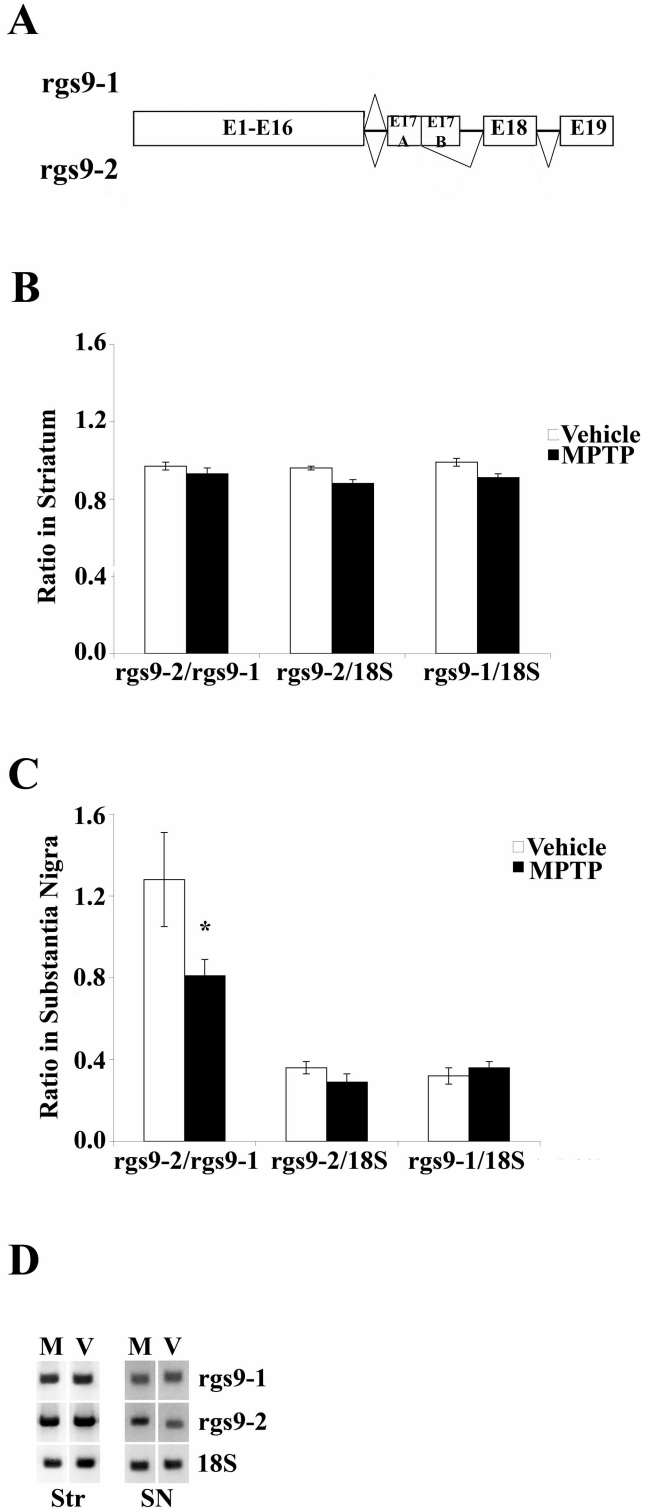
fosB
ΔfosB
18S
Str
SN


A
rgs9-1
rgs9-2
E1-E16
E17 A
E17 B
E18
E19
B
Ratio in Striatum
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1.2
0.8
0.4
0.0
Vehicle
MPTP
rgs9-2/rgs9-1
rgs9-2/18S
rgs9-1/18S
C
Ratio in Substantia Nigra
1.6
1.2
0.8
0.4
0.0
Vehicle
MPTP
*
rgs9-2/rgs9-1
rgs9-2/18S
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D
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M V
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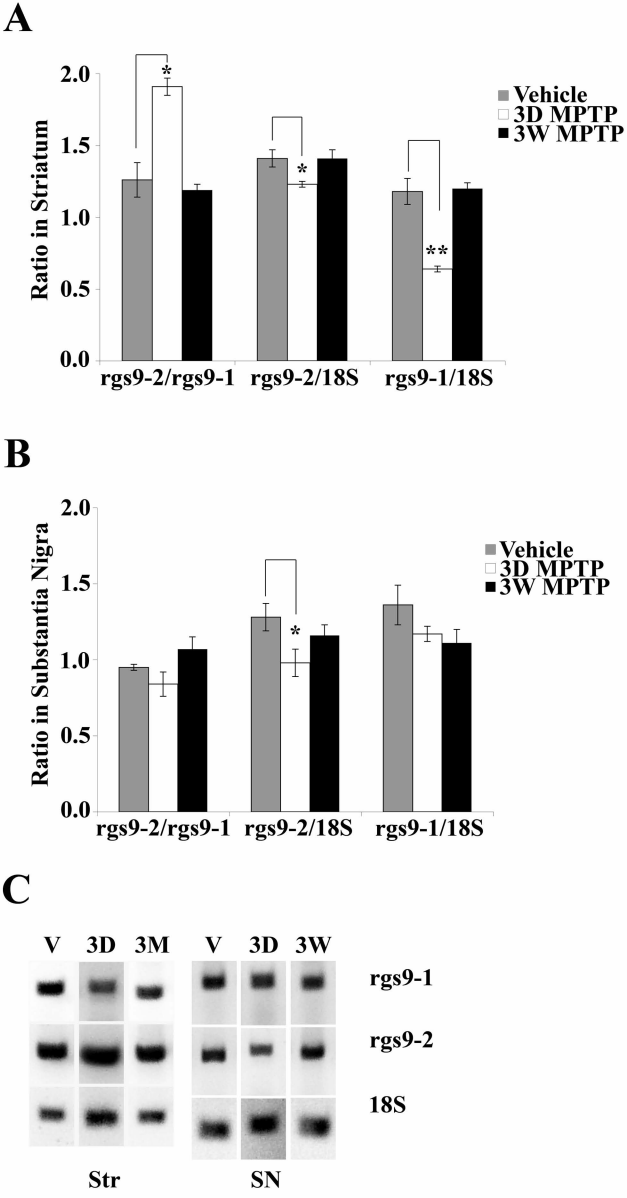
18S
Str
SN


A
2.0
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Ratio in Striatum
*
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**
Vehicle
3D MPTP
3W MPTP
rgs9-2/rgs9-1
rgs9-2/18S
rgs9-1/18S
B
2.0
1.5
1.0
0.5
0.0
Ratio in Substantia Nigra
*
Vehicle
3D MPTP
3W MPTP
rgs9-2/rgs9-1
rgs9-2/18S
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Str
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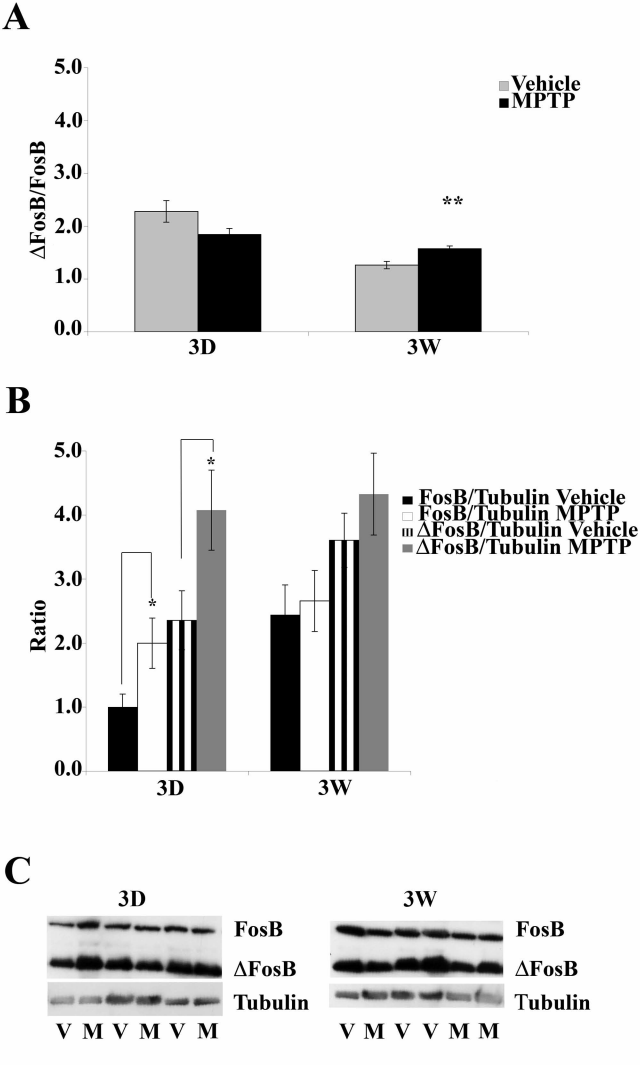
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4.0
3.0
2.0
1.0
0.0
ΔFosB/FosB
Vehicle
MPTP
**
3D
3W
B
5.0
4.0
3.0
2.0
1.0
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Ratio
*
*
FosB/Tubulin Vehicle
FosB/Tubulin MPTP
ΔFosB/Tubulin Vehicle
ΔFosB/Tubulin MPTP
3D
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3D
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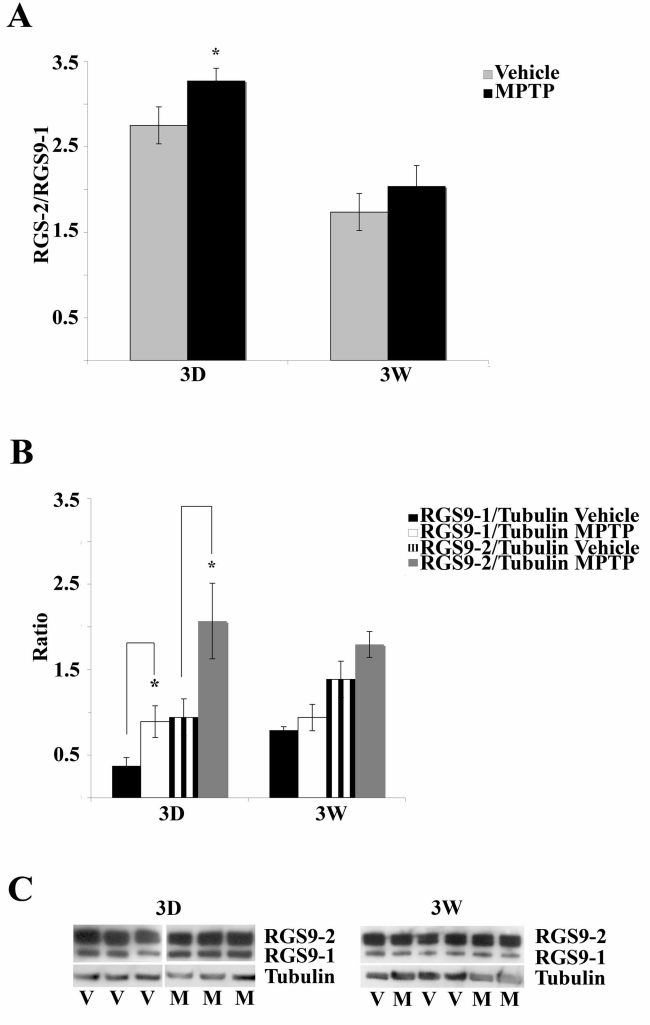
FosB
ΔFosB
Tubulin
FosB
ΔFosB
Tubulin
V M V M V M
V M V V M M


A
RGS-2/RGS9-1
3.5
2.5
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*
Vehicle
MPTP
3D
3W
B
Ratio
3.5
2.5
1.5
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*
*
RGS9-1/Tubulin Vehicle
RGS9-1/Tubulin MPTP
RGS9-2/Tubulin Vehicle
RGS9-2/Tubulin MPTP
3D
3W
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3D
3W
RGS9-2
RGS9-1
Tubulin
V V V M M M
RGS9-2
RGS9-1
Tubulin
V M V V M M

FOSB AND RGS9 SPLICE VARIANTS ARE BIOMARKERS FOR MPTP EXPOSURE

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Abbreviations

AChE	acetylcholinesterase
AR-JP	autosomal recessive juvenile parkinsonism AR-JP
DA	dopamine
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FTDP-17	frontotemporal dementia and Parkinsonism linked to chromosome 17
g	grams
i.p.	intraperitoneal
kg	kilograms
mg	milligrams
ml	milliliter
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PD	Parkinson's disease
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous

Abstract

Most cases of Parkinson's disease are sporadic, suggesting environmental toxins may be an instigating factor in disease development. Currently, there are no biomarkers to identify susceptible individuals. Changes in gene expression are ideal biomarkers since these occur rapidly in response to environmental stimuli. Using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse models, we identified changes in the splicing of fosB and rgs9 pre-mRNAs in the blood. The ratio of fosB/18S rRNA and rgs9-2/18S rRNA transcripts decreased after acute MPTP treatment. Additionally, the ΔfosB/18S rRNA and rgs9-2/rgs9-1 transcript ratios decreased after chronic treatment. These data suggest that splice variants of fosB and rgs9 may be useful as biomarkers, since they indicate the immediate and long-term responses to the physiological stress associated with Parkinsonism.

Keywords: Parkinson's disease, pre-mRNA splicing, MPTP, FosB, RGS9, neurodegeneration

Introduction

The pathological hallmarks of Parkinson's disease (PD) are the progressive and selective loss of nigrostriatal dopamine (DA) neurons and the presence of proteinaceous cytoplasmic inclusions called Lewy bodies (Forno, 1996, Dauer, 2003). The mechanisms underlying PD development and progression have not been fully characterized, although increasing evidence indicates that mitochondrial dysfunction, oxidative damage, excitotoxicity, and inflammation are contributing factors (Dunnett and Bjorklund, 1999, Vila et al., 2000, Dawson and Dawson, 2002, 2003, Hunot and Hirsch, 2003, Meredith, 2004).

Approximately 5% of PD patients have inherited a genetic mutation that makes them susceptible to developing the disease. Ten genes have been identified that are involved in the familial forms of the disease (reviewed by Dawson and Dawson, 2003). Patients who have inherited a familial form of PD are rare, however, and most cases are idiopathic suggesting environmental factors play an important role in disease development. The basis for this theory comes from the knowledge that environmental toxins often act to inhibit mitochondrial function and mitochondrial dysfunction has been associated with PD (Benecke et al., 1993, Schapira et al., 1993, Haas et al., 1995, Beal, 2003). There is also strong experimental support for the idea that toxic environmental factors can combine with a genetic susceptibility for the development of PD (Huang et al., 2004). Oxidative stress can damage a cell's complement of proteins and DNA and nigral DA neurons are particularly sensitive to this type of insult (Jenner, 1998). Additionally, reactive oxygen species (ROS) are associated with Lewy bodies (Meredith, 2004, Terman and Brunk, 2004). Understanding the susceptibility of an individual to PD

clearly requires knowledge of both the genetic processes and environmentally-induced oxidative stressors that can damage DA neurons**.** In this regard, many neurological diseases are associated with the dysregulation of splicing (for reviews see D'Souza et al., 1999, Grabowski and Black, 2001, Zhang et al., 2002). One example is autosomal recessive juvenile parkinsonism (AR-JP) which involves the gene that encodes parkin. The parkin mRNA in leukocytes from AR-JP patients was shorter than that in the brain due to splicing that results in the exclusion of exons 3,4 and 5 from the mature transcript (Sunada et al., 1998). Another example is inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), which is associated with intraneuronal tau-containing deposits. In FTDP-17, multiple mutations in the tau gene can alter the regulated splicing of the transcript (Hutton et al., 1998, D'Souza et al., 1999). Results from these studies indicate that small changes in the ratio of splice products may underlie the neurodegenerative process, and emphasize the importance of tightly maintaining ratios of various splice isoforms.

Currently, the number of studies that have examined changes in splicing in animal models of PD is very limited. In a MPTP mouse model, overexpression of one splice variant of the acetylcholinesterase gene, AChE-R, was protective, whereas overexpression of another variant, AChE-S, enhanced the development of Parkinsonism (Ben-Shaul et al., 2006). In another study, a shift in splice variants from AChE-S to AChE-R was associated with neurodegeneration and stress-associated disorders (Meshorer and Soreq, 2006).

Identification of splice variants as biomarkers for PD in an easily obtained body fluid such as blood may be a means of determining exposure to environmental toxins in

patients that may place them at greater risk for developing the disease in the long term. In this study we show that the amount of fosB and rgs9 splice variants in the blood of mice reflect their acute and/or chronic exposure to MPTP. The amount of fosB and rgs9-2 transcripts normalized to 18S rRNA in the blood decreased after acute MPTP treatment. In addition, the ΔfosB/18S rRNA and rgs9-2/rgs9-1 transcript ratios decreased after chronic MPTP/probenecid treatment. These results indicate that monitoring the splice variants of fosB and rgs9 in blood may be an indicator of the immediate and long-term responses to environmental factors that play a role in the development of Parkinsonism.

Experimental Procedures

Animals. C57BL/6 male mice weighing 18-22g (Charles River Laboratories, Wilmington, MA, USA) were housed on the average of three per cage with food and water available *ad libitum*. During the injection protocol, mice are also given ~15 ml of Harlan transit gel (Harlan Sprague Dawley, Indianapolis, IN) every day to keep them well hydrated. All animal protocols were approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee. Animal protocols were also in accord with the U.S. Public Health Service Policy and the National Institutes of Health Guide on the Humane Care and Use of Laboratory Animals.

One group of mice received an acute treatment of 4 injections of MPTP (20 mg/kg in saline, i.p.) every 2 hours and 3 days post-treatment the mice were euthanized by cervical dislocation. For the chronic treatment protocol, mice were injected with MPTP (25 mg/kg in saline, s.c) and probenecid (250 mg/kg, i.p.) every 3.5 days for 5 weeks (total of 10 doses or 250mg/kg MPTP) (Petroske et al., 2001). Either 3 days or 3

weeks after treatment, all mice were euthanized by cervical dislocation. The controls for both treatments were injected with saline using the same protocols.

RNA Preparation and Amplification by Polymerase Chain Reaction. Following cervical dislocation, a heart puncture was used to obtain blood with a syringe pre-treated with 0.05M EDTA. The blood was dispensed into 0.2ml aliquots and immediately flash frozen in liquid nitrogen. Total RNA was extracted from whole blood using the TRI-Reagent BD system as per the manufacturer's instructions (Molecular Research Center, Inc, Cincinnati, OH). The RNA pellet was resuspended in 60 ml of nuclease-free water, heated to 65^{o}C for 10 min and then stored at -80 °C. Reverse Transcriptase PCR (RT-PCR) was carried out as previously described (J.A. Potashkin, U. J. Kang, P. A. Loomis, F. M. Jodelka, Y. Ding, G. E. Meredith, submitted). Microsoft Excel 2004 for Mac version 11.0 software (Bellevue, WA) was used to make statistical comparisons among groups using a t-test analysis for two samples assuming unequal variance. Differences with a probability of error of less than 5% were considered statistically significant.

Results

In this study, one group of mice was treated acutely with MPTP and RNA was prepared from blood after the animals were euthanized. Semi-quantitative RT-PCR was used to amplify the fosB, rgs9 and rRNA (control) transcripts. We examined two splice variants of *fosB* produced by the retention or splicing of intron 4, fosB or Δfos B, respectively (Fig. 1A). The results showed that there was a significant decrease in the fosB/18S rRNA ratio after acute MPTP treatment compared to saline treated mice (Fig. 1B, $p<0.01$ and

1C). We also examined two splice variants of rgs9 produced by the retention or splicing of exon 17B and intron 17, rgs9-1 and rgs9-2, respectively (Fig. 2A). There was a significant decrease in the rgs9-2/ 18S rRNA ratio in the MPTP treated mice compared to controls (Fig. 2B, $p<0.05$ and 2C).

In another series of experiments, mice were treated chronically with MPTP/probenecid and then euthanized either 3 days or 3 weeks post-treatment. Mice treated chronically with MPTP had significantly lower dopamine concentrations in the striatum, pronounced cell death of TH-immunopositive neurons in the substantia nigra pars compacta at 3 days and 3 weeks after treatment and a significant decrease in motor performance in a grid test compared to vehicle treated controls (Petroske et al., 2001, Chan et al., 2007) (J.A. Potashkin, U. J. Kang, P. A. Loomis, F. M. Jodelka, Y. Ding, G. E. Meredith, submitted). The results from the RT-PCR studies showed that there was no significant change in the fosB (Figs. 3A and B) or rgs9 (Figs. 4A and B) splice variants 3 days after termination of treatment. There was, however, a significant decrease in the ΔfosB/ 18S rRNA compared to controls 3 weeks after MPTP treatment (Fig. 3A, $p<0.05$ and 3B). In addition, there was a significant decrease in the rgs9-2/rgs9-1 ratio in the MPTP treated mice compared to controls (Fig. 4A, $p<0.05$ and 4B).

Discussion

A positive aspect to the tragic circumstances that resulted in the discovery that MPTP can produce Parkinsonism in humans (Langston et al., 1983) has been the wealth of data obtained through the use of this toxin to decipher the cytopathology intrinsic to this devastating neurological disorder. Indeed, the mouse MPTP model has become

particularly useful for studying the effects of short- and long-term exposure to that toxin. The acute administration of MPTP to mice rapidly destroys many dopaminergic neurons of the nigrostriatal pathway and produces a Parkinsonian syndrome similar to PD (Przedborski and Vila, 2003, Meredith and Kang, 2006). Probenecid slows the metabolism of MPTP and thus chronic administration of both together reduces nigrostriatal function to a greater extent than protocols that use MPTP alone (Petroske et al., 2001).

Tragically, when symptomatic individuals seek medical treatment, usually 70-80% of the neurons in the substantia nigra pars compacta have been lost. Biomarkers to identify individuals at a pre-symptomatic stage would be beneficial for identifying toxic environmental factors whose continued exposure may augment the progression of PD. In this study, we used both the acute and chronic MPTP protocols to characterize the splice variants of fosB and rgs9 pre-mRNAs in blood of mice to determine if they might be useful as biomarkers for exposure to environmental toxins that could lead to PD-like pathology in the brain. Our results suggest that the splice variants of fosB and rgs9 may indeed be useful markers since the fosB and rgs9-2 transcripts decreased in the blood after acute exposure and the ratios of ΔfosB/ 18S rRNA and rgs9-2/rgs9-1 decreased 3 weeks after chronic exposure to MPTP. Thus, monitoring splice variants in blood is a means to assess the effects of both the short- and long-term exposure to toxins that play a role in the development of Parkinsonism.

ΔFosB and RGS9 proteins are involved in dopamine signaling (Hope et al., 1994, Doucet et al., 1996, Gold et al., 1997, Perez-Otano et al., 1998, Cenci et al., 1999, Rahman et al., 1999, Rahman et al., 2003). Postmortem examination of brain sections

found both proteins are more abundant in the striatum of PD patients compared to controls (Tekumalla et al., 2001). In addition, experimental models of PD and L-DOPA-induced dyskinesia are characterized by increased ΔFosB expression in the striatum compared to controls (Perez-Otano et al., 1998, Cenci, 2002, Pavon et al., 2006). ΔFosB is a truncated protein compared to FosB and the loss of the carboxy-terminus makes it more stable (Nestler et al., 2001). Because of its stability, ΔFosB tends to accumulate with time in response to chronic stimuli (Nestler et al., 2001, McClung et al., 2004). After dopamine denervation and L-dopa treatment, ΔFosB is induced and may form heterodimers with JunD that bind to CRE sites and block the binding of CREB (Andersson et al., 2001). In addition to its activity at CRE sites, high levels of ΔFosB can activate transcription at AP-1 sites after forming heterodimers with Jun (Dobrazanski et al., 1991, Chen et al., 1997, Chen et al., 2000). The splicing of fosB was recently investigated in vivo and the results showed that the expression of both fosB and ΔfosB increase in the striatum after acute amphetamine administration or stress (Alibhai et al., 2007). In the same study, chronic administration of amphetamine did not produce a significant change in the ratio of ΔfosB to fosB in the striatum. Interestingly, in the blood we observed a decrease in ΔfosB after chronic MPTP treatment and a decrease in fosB after acute MPTP administration. The consequences of the downregulation of both splice variants with regards to gene expression is unknown but will likely involve expression at AP-1 sites and perhaps additionally CRE sites. These results suggest that MPTP may produce short- and long-term changes that alter transcription dynamics.

RGS9 is a regulator of G proteins that suppresses signaling efficacy of G-protein-coupled receptors (Dohlman and Thorner, 1997). RGS9-2, is enriched in the striatum and

is also present in the substantia nigra (Kim et al., 2005). RGS9-2 suppresses the $G_{i/o}$-coupled μ opioid receptor response (Rahman et al., 1999). RGS9-1 is abundantly expressed in the retina and is believed to be a GTPase-activating protein for transducin (He et al., 1998, Chen et al., 2000). The main structural difference between RGS9-1 and RGS9-2 is their carboxy-terminal regions that are produced by alternative splicing. This region of the proteins is important for their subcellular localization. RGS9-1 is mainly localized to the retinal rod outer segment membrane, while RGS9-2 is predominately localized to neuronal nuclei in the brain (He et al., 2001, Bouhamdan et al., 2004). The altered expression of these two splice variants of RGS9 on the pathophysiology of Parkinson's disease remains unknown.

Acknowledgements

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Figure Legends

Figure. 1. In vivo expression of fosB splice isoforms in the blood after acute MPTP treatment. Alternative splicing of fosB pre-mRNA produces a □fosB and fosB mRNA by the splicing or retention, respectively, of intron 4 (A). Mice were treated acutely with MPTP and RNA from the blood was analyzed 3 days after the last injection (B). Data are expressed as mean±sem (n=5). A representative gel showing the products of the PCR reactions (C). V, vehicle; M, MPTP

Figure. 2. In vivo expression of rgs9 splice isoforms in the blood after acute MPTP treatment. Alternative splicing of rgs9 pre-mRNA produces an rgs9-1 and rgs9-2 mRNA by the inclusion of the entire exon17 or exon17A, exon18 and exon19, respectively (A). Mice were treated acutely with MPTP and RNA from the blood 3 days after the last injection (B). Data are expressed as mean±sem (n=5). A representative gel showing the products of the PCR reactions (C). V, vehicle; M, MPTP

Figure. 3. In vivo expression of fosB splice isoforms in the blood after chronic MPTP and probenecid treatment. Mice were treated chronically with MPTP and RNA from the blood was analyzed three days (3D) or three weeks (3W) after the last injection (A). Data are expressed as mean±sem (n=9 saline, 16 MPTP). A representative gel showing the products of the PCR reactions (B).

Fig. 4. In vivo expression of rgs9 splice isoforms in the blood after chronic MPTP treatment. Mice were treated chronically with MPTP and RNA from the blood (B) three days (3D) or three weeks (3W) after the last injection (A). Data are expressed as mean±sem (n=9 saline and 16 MPTP). A representative gel showing the products of the PCR reactions (B).

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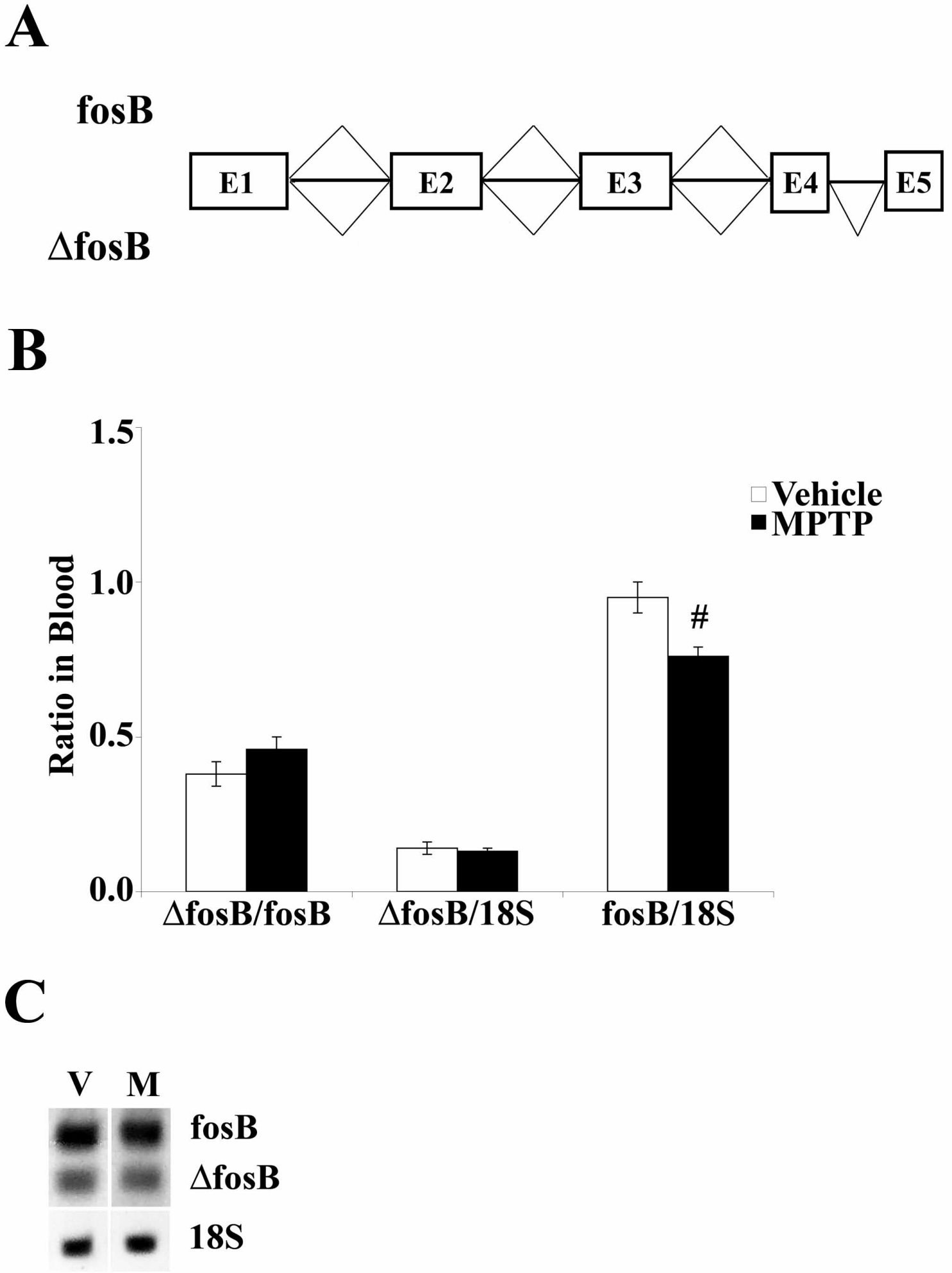
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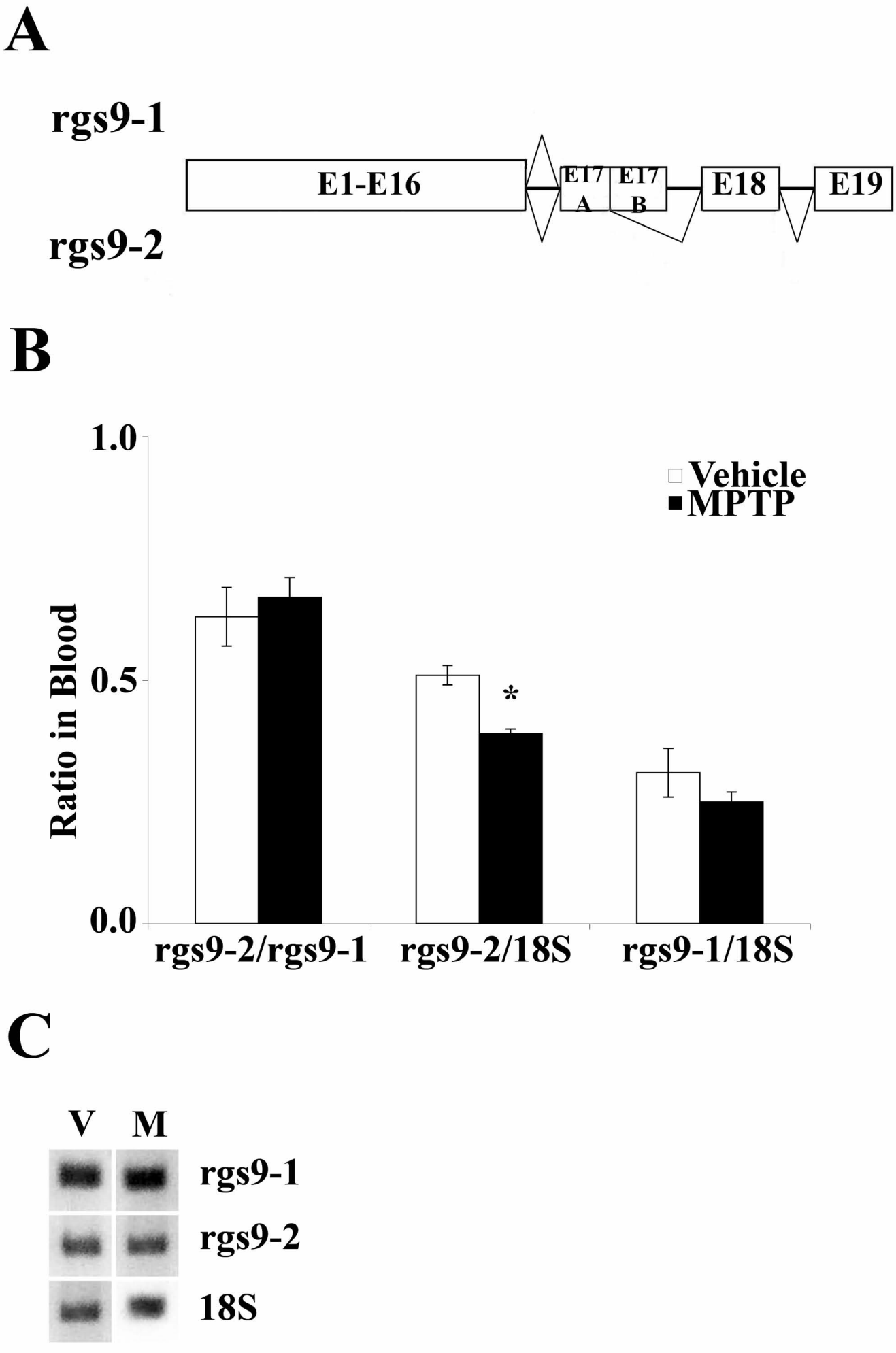
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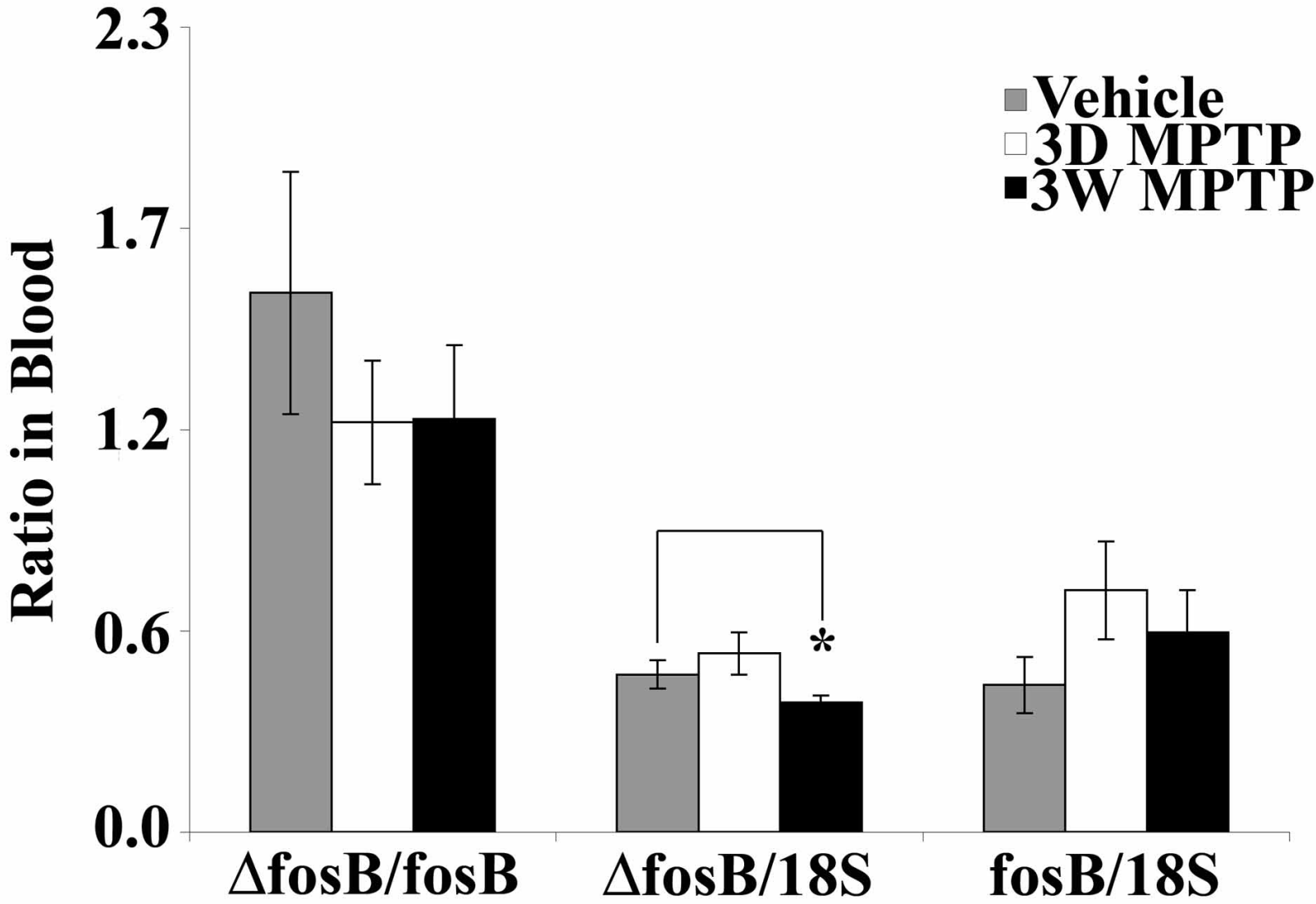
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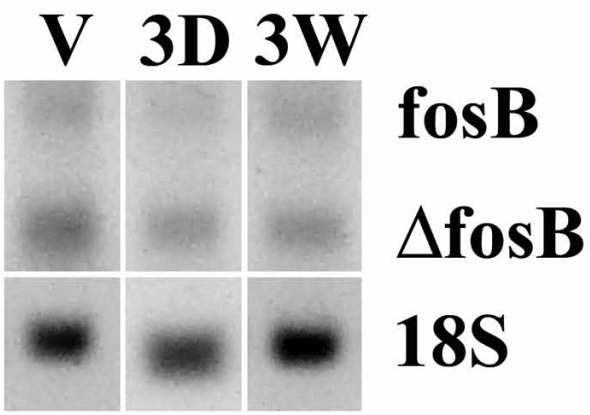
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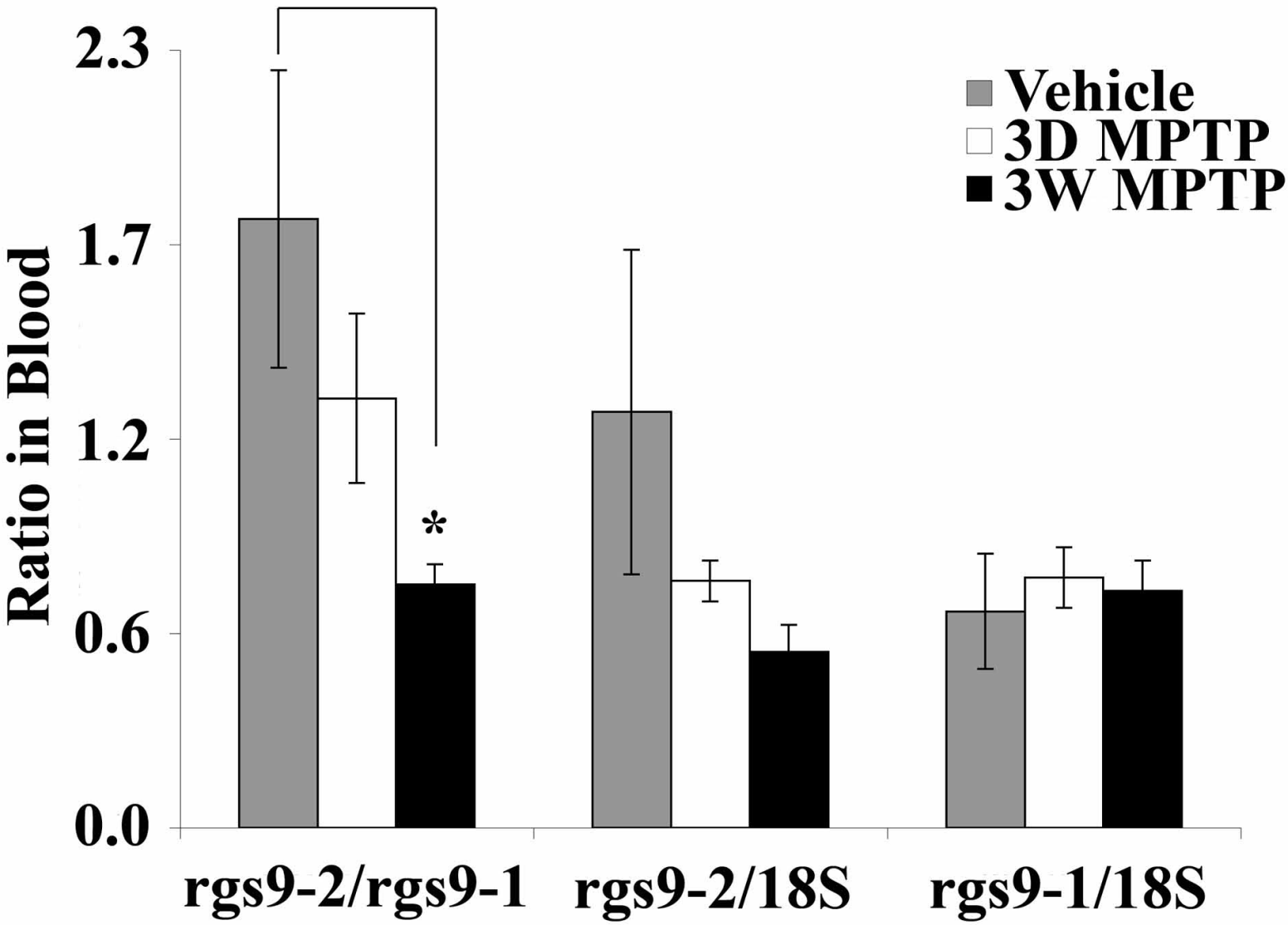
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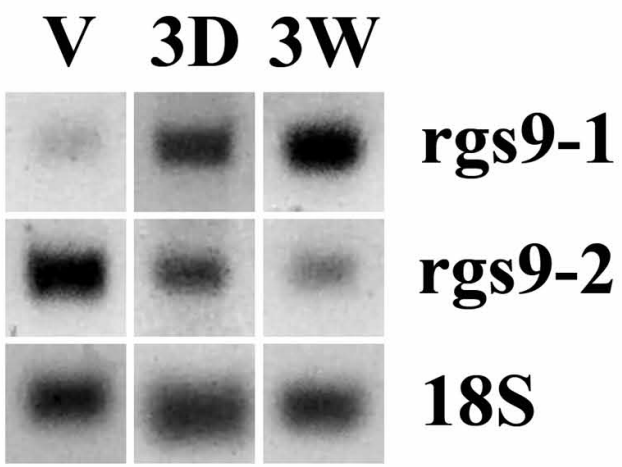
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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF

MCMR-ZB-P 30 Aug 2007

MEMORANDUM FOR THE RECORD

SUBJECT: Initial Approval for the Protocol, "Identification of Splice Variants as Molecular Markers in Parkinson's Disease," Submitted by Gloria E. Meredith, PhD, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois, Proposal Log Number 05090011, Award Number W81XWH-05-1-0580, HRPO Log Number A-13416

1. The subject protocol (received 31 July 2007) was approved by the University of Chicago Institutional Review Board (IRB) 4 August 2006 with a continuing review approved on 3 August 2007, by the Medical College of Wisconsin IRB on 22 January 2007, and by the Rosalind Franklin University IRB on 30 August 2006 with a continuing review approved on 18 July 2007. This protocol was reviewed by the U.S. Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) and found to comply with applicable Federal, DOD, U.S. Army, and USAMRMC human subjects protection requirements.

2. This no greater than minimal risk study is approved for enrollment of 100 subjects.

3. Please note the following reporting obligations:

a. Major modifications to the research protocol and any modifications that could potentially increase risk to subjects must be submitted to the USAMRMC ORP HRPO for approval prior to implementation. All other amendments must be submitted with the continuing review report to the HRPO for acceptance.

b. All unanticipated problems involving risks to subjects or others, serious adverse events related to study participation, and deaths related to study participation must be reported to the HRPO.

c. Any deviation to the subject protocol that affects the safety or rights of the subject and/or integrity of the study data must be reported promptly to the HRPO.

d. All modifications, deviations, unanticipated problems, adverse events, and deaths must also be reported at the time of continuing review of the protocol.

e. A copy of the continuing review reports approved by the University of Chicago IRB, the Medical College of Wisconsin IRB, and the Rosalind Franklin IRB must be submitted to the HRPO as soon as possible after receipt of approval. It appears the next continuing review by the University of Chicago IRB is due no later than 2 August 2008, the Medical College of Wisconsin IRB is due no later than 21 January 2008, and the Rosalind Franklin IRB no later than 21 January 2008.

f. In addition, the current version of the protocol and consent forms must be submitted along with the continuing review reports and the University of Chicago IRB, the Medical College of Wisconsin IRB, and the Rosalind Franklin IRB approval notices for continuation of the protocol.

g. The final study reports submitted to the University of Chicago IRB, the Medical College of Wisconsin IRB, and the Rosalind Franklin IRB, including copies of any acknowledgement documentation and any supporting documents, must be submitted to the HRPO as soon as all documents become available.

4. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grants Officer can authorize expenditure of funds. It is recommended that you contact the appropriate contract specialist or contracting officer regarding the expenditure of funds for your project.

5. The HRPO point of contact for this study is Kristen R. Katopol, MS, CIM, Human Subjects Protection Scientist, at 301-619-1119/Kristen.Katopol@us.army.mil.

E-Signed by LAURA R BROSCH
VERIFY authenticity with Approvel

LAURA R. BROSCH, PhD
Colonel, Army Nurse Corps
Director, Office of Research Protections